ABSTRACT

Title of Thesis: INFLUENCE OF VITAMIN D RECEPTOR GENE POLYMORPHISMS ON CHANGES IN INSULIN SENSITIVITY WITH AEROBIC EXERCISE TRAINING

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The purpose of the study was to examine the influence of the FokI and BsmI polymorphisms of the vitamin D receptor gene on changes in insulin sensitivity with aerobic exercise training in men and women at increased risk for type 2 diabetes mellitus (T2DM). Subjects were genotyped and underwent oral glucose tolerance tests before and after six months of training. Due to mis-genotyping of the BsmI polymorphism, results for that variant were not reported. There were no significant differences between FokI genotype groups in insulin sensitivity before or after training. However, among subjects who completed training, FF homozygotes had significantly higher baseline fasting glucose and insulin levels than f allele carriers. While the FokI polymorphism does not appear to mediate training-induced changes in indices of glucose and insulin metabolism, it may influence fasting glucose and insulin levels and the development of insulin resistance in individuals at increased risk for T2DM.

INFLUENCE OF VITAMIN D RECEPTOR GENE POLYMORPHISMS ON CHANGES IN INSULIN SENSITIVITY WITH AEROBIC EXERCISE TRAINING

by

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List of Abbreviations

$1,25(OH)_2D_3$: 1,25-dihydroxyvitamin D_3

AHA: American Heart Association

AUC: Total area under the response curve

BMI: Body mass index

BP: Blood pressure

FPG: Fasting plasma glucose

FPI: Fasting plasma insulin

GERS: Gene Exercise Research Study

HRT: Hormone replacement therapy

IAAT: Intra-abdominal adipose tissue

ISI: Insulin sensitivity index

IVGTT: Intravenous glucose tolerance test

mRNA: Messenger ribonucleic acid

OGTT: Oral glucose tolerance test

SCAT: Subcutaneous adipose tissue

SE: Standard error

T2DM: Type 2 diabetes mellitus

VDR: Vitamin D receptor

VDRE: Vitamin D response element

VO_2max: Maximal oxygen uptake

CHAPTER 1: INTRODUCTION

Background

Diabetes mellitus is a group of metabolic diseases of significant public health importance, as it is the fifth leading cause of death by disease in the United States (71). The most prevalent form of diabetes, type 2 diabetes mellitus (T2DM), accounts for 90-95% of all diagnosed cases of diabetes, and compared to nondiabetics, individuals with T2DM are at a significantly greater risk for a number of micro- and macrovascular complications, including retinopathy, peripheral and autonomic neuropathy, nephropathy, peripheral vascular disease, atherosclerotic cardiovascular and cerebrovascular disease, hypertension, susceptibility to infections, and periodontal disease (1).

T2DM is a complex trait associated with genetic, environmental, and metabolic defects. Central to its pathophysiology are the key defects of insulin resistance and impaired insulin secretion (1,30,58). One environmental factor that has been associated with insulin resistance and impaired insulin secretion is vitamin D deficiency (13). Vitamin D deficiency has been associated with insulin resistance in humans (8) and with impaired insulin secretion in both experimental animals and in humans (13,14,48,63), while vitamin D supplementation has been associated with improved insulin secretion in vitamin D deficient rats (17,18,48) and in type 2 diabetics (12,39).

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], acts through the vitamin D receptor (VDR), which regulates gene transcription by binding to vitamin D response elements (VDRE) in the promoter

region of target genes (29,36). The VDR is expressed in pancreatic beta cells (35), and in mice with nonfunctioning VDR, oral glucose tolerance and insulin secretion are impaired, demonstrating a molecular role of the VDR in pancreatic endocrine function (89). In addition, several polymorphisms of the gene encoding the VDR have been investigated in association with insulin resistance, insulin secretion, and T2DM. In humans, the VDR FokI polymorphism has been associated with insulin sensitivity (20), and the VDR BsmI polymorphism has been associated with insulin resistance (64), pancreatic beta cell secretory capacity (79), and T2DM risk (65), although not all investigations have shown an association with the BsmI variant (33,86).

An additional environmental factor that has been associated with insulin resistance and T2DM is physical activity. The classification of habitual physical inactivity as a risk factor for T2DM (71) is supported by a number of epidemiologic studies that have investigated the association between level of physical activity and incidence of T2DM. These studies demonstrate a reduced risk of T2DM with increasing physical activity, especially among individuals at high risk for the disease (22,28,32,54,55,81). Additionally, insulin sensitivity is increased in exercise-trained individuals (28), and regular physical activity is an important therapeutic modality for T2DM, as it has been shown to improve insulin sensitivity in type 2 diabetics (1).

Statement of the Problem

Considering the public health impact of T2DM, the importance of the prevention of the disease is evident, especially in those at increased risk. There is evidence that genes (16,58), as well as physical activity level, influence the

development of insulin resistance, impaired insulin secretion, and T2DM. Insulin sensitivity, which has a modest heritability (84) ranging from 28% to 54% in nondiabetics (23,38,50,59,76,84), is also influenced by physical activity level; however, only one study has accounted for the effects of exercise training status on the association between the VDR gene and insulin sensitivity (26). In a complex disease such as T2DM, it is important to not only identify the susceptibility genes that place an individual at an increased risk, but also the environmental factors, such as physical activity, that may attenuate the risk associated with those genes. As such, the purpose of the present study was to evaluate the association of the FokI and BsmI polymorphisms of the VDR gene with changes in insulin sensitivity as a result of aerobic exercise training. It was hypothesized that:

1. For the FokI polymorphism, before training, f allele carriers would have lower insulin sensitivity than FF homozygotes. After training, f allele carriers would exhibit a greater increase in insulin sensitivity compared to FF homozygotes.
2. For the BsmI polymorphism, before training, b allele carriers would have lower insulin sensitivity than BB homozygotes. After training, b allele carriers would exhibit a greater increase in insulin sensitivity compared to BB homozygotes.

CHAPTER 2: METHODS

The present study was conducted as a sub-study within the Gene Exercise Research Study (GERS) at the University of Maryland, College Park. GERS was designed to describe the relationship between specific gene polymorphisms and aerobic exercise training-induced improvements in blood lipids and blood pressure (BP). While many of the methods were optimized for evaluation of the GERS hypotheses, many aspects of the study design and methods, such as screening methods, dietary stabilization, and weight loss restrictions, serve well for examining insulin sensitivity. In addition, subjects recruited for GERS were at an increased risk for type 2 diabetes mellitus (T2DM), as they were greater than 45 years of age and sedentary. Some subjects possessed additional risk factors for T2DM, such as overweight (body mass index $\geq$25 kg/m^2), African-American, Hispanic-American, or Asian-American/Pacific Islander ethnicity, hypertension ($\geq$140/90 mm Hg), and/or dyslipidemia (HDL-cholesterol $\leq$35 mg/dL and/or a triglyceride level $\geq$250 mg/dL) (5); thus, these were important individuals to examine in relation to gene-exercise interactions with insulin sensitivity.

The Institutional Review Board (IRB) at the University of Maryland, College Park approved all procedures as described in the IRB application for the GERS project. Written approval for the use of GERS data in the present study was obtained from the IRB.

Sample Sizes

VDR FokI and BsmI genotypes were obtained for 120 and 119 sedentary subjects, respectively. Complete oral glucose tolerance test (OGTT) data was

obtained from 106 of those subjects. Of the sedentary subjects, 77 with FokI genotypes and 76 with BsmI genotypes completed six months of aerobic exercise training and were re-tested in the trained state. Complete OGTT data was obtained from 70 of those trained subjects.

Subject Recruitment

Volunteer men and women for the present study were recruited from College Park, Maryland and the surrounding areas through advertising such as radio public service announcements, newspaper advertisements and columns, and direct mailings. Advertisements targeted individuals who were 50-75 years of age, sedentary, postmenopausal, and free from cardiovascular disease and diabetes. The advertisements encouraged interested individuals to call the GERS study office for more information about the study and to initiate screening.

Subject Screening

Screening was used to ensure that enrolled subjects could safely complete the study testing and intervention, and to enroll subjects who were appropriate for optimal evaluation of the GERS hypotheses. The screening consisted of questions administered over the telephone and three visits to the College Park site.

Potential GERS subjects were given a brief description of the study and screened for provisional acceptance into the study over the telephone. A telephone interview was used to select sedentary (regular aerobic exercise $\leq$2 times per week and <20 minutes per session; sedentary occupation), 50-75 year old non-smokers with a body mass index (BMI) of 37 kg/m^2 or less. BMI had to be 37 kg/m^2 or less so that the physical limitations of excessive obesity would not impede subjects' ability to

exercise train vigorously. Potential subjects also had to report that they were free from metabolic, hematological, cardiovascular, and major organ diseases and could not be taking lipid- or glucose-lowering medications. Self-reported BP had to be less than ~160/100 mm Hg; however, only individuals reporting substantially higher BPs than this were excluded via telephone screening. If on antihypertensive medications, only individuals taking antihypertensive medications not known to affect lipid or glucose metabolism qualified. Additionally, those taking antihypertensive medication had to be willing to taper off of the medication. Potential subjects also had to be free from orthopedic conditions that could have affected their ability to tolerate vigorous exercise. Women had to be at least two years postmenopausal and had to agree to maintain their hormone replacement regimen, either on or not on, constant for the duration of the study.

Those subjects provisionally qualified based on the telephone screening were scheduled for an orientation meeting, in which they visited the College Park facility to give their informed, written consent to participate in the study. Additionally, subjects had their health history and level of daily physical activity reviewed to ensure they qualified for the study. Subjects then underwent two on-site screening appointments to select those who were qualified for GERS. Qualified subjects had to have clinical dyslipidemia (25) or pre-hypertension or stage 1 hypertension (21).

At the first screening appointment, subjects had their height and weight measured and BMI calculated to ensure that it was <37 kg/m^2. Subjects also had their resting BP measured according to the guidelines of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (21). A

blood sample was drawn after a 12-hour overnight fast for genotyping and for screening blood chemistries. Subjects were excluded from the study if they had hematocrit <35 or evidence of renal or liver disease. Fasting plasma glucose, which had to be <126 mg/dL for subjects to qualify, was also evaluated. Subjects then ingested 75 g of a flavored dextrose solution for a diagnostic OGTT. Glucose measured two hours later had to be <200 mg/dL for subjects to qualify.

At the second screening appointment, subjects qualified on the basis of screening blood chemistry and OGTT had a physical and cardiovascular examination by a physician to detect cardiovascular, pulmonary, or other chronic disease that would prevent exercise testing or training. Subjects underwent a graded exercise test using the Bruce treadmill protocol to ensure that they had no evidence of overt cardiovascular disease. Blood pressure, heart rate, and electrocardiogram were recorded before the test, at the end of every exercise stage, and every other minute for six minutes after exercise. The test was terminated when the subject could no longer continue or cardiovascular signs or symptoms developed. Subjects had to have <2 mV ST-segment depression and no cardiovascular signs or symptoms during the treadmill exercise test to be included in the study.

Dietary Stabilization and Drug Tapering

Qualified subjects then entered the dietary stabilization phase of the protocol. All qualified subjects were advised to consume a standard diet that met the American Heart Association (AHA) Dietary Guidelines for the General Population (47). Six weeks of dietary classes preceded baseline testing. The 45-minute classes were held twice a week and were taught by a registered dietician. Only subjects who were

compliant with the AHA guidelines as determined from a 7-day food record and computerized nutrient analysis (Nutritionist IV, N-Squared Computing, Salem Park, Oregon) progressed to baseline testing. Subjects were encouraged to maintain the AHA recommended diet throughout the study. To increase compliance with the diet, subjects completed food frequency questionnaires at two time points during the exercise training intervention. A registered dietician evaluated the completed food frequency questionnaires and the results were used as a basis for dietary consultation with subjects. At the end of the exercise training intervention, subjects completed another 7-day diet record, which was also evaluated for compliance with the AHA guidelines. Subjects demonstrating large changes in diet during the exercise training intervention as indicated by a comparison of the initial and final 7-day food records were excluded from analysis. In addition, subjects were weighed at each dietary class and then once a week throughout the exercise training intervention. Those losing weight were counseled to increase their caloric intake back to its initial level. If changes in body weight occurred during the aerobic exercise training intervention that were greater than that expected from the exercise energy expenditure alone, those subjects were excluded from analysis.

Subjects in the BP sub-study of GERS were tapered off of antihypertensive medication, if applicable, during the last four weeks of the dietary stabilization period under the close direction of the GERS staff and the subjects' personal physicians. During tapering, BP was monitored during each dietary class to ensure that subjects' BP did not rise above 159 mm Hg or 99 mm Hg for systolic and diastolic BP, respectively. If BP rose above these values for three consecutive weeks, those

subjects were excluded from the study and were referred back to their personal physicians for treatment.

Aerobic Exercise Training Intervention

Exercise training consisted of six months of three sessions per week, supervised aerobic exercise training using indoor exercise equipment such as treadmills, stationary bicycles, rowing machines, stair steppers, and elliptical trainers. Subjects used heart rate monitors to control exercise intensity, and all exercise sessions started and concluded with appropriate warm-up and cool-down activities. Over the first 10 weeks of the program, duration and intensity of exercise were gradually increased. Initial training sessions consisted of 20 minutes of exercise at 50% VO_2max. Training duration increased by 5 minutes every week until 40 minutes of exercise at 50% VO_2max was completed each session. Training intensity was then increased by 5% VO_2max every week until an intensity of 70% VO_2max was achieved. Increases in training duration or intensity occurred only if subjects completed their exercise prescription for three consecutive sessions without cardiovascular signs or symptoms or undue fatigue. In addition, starting at week 10 of exercise training, subjects were asked to add one unsupervised 45-60 minute moderate-intensity bout of exercise to their weekly routine. Subjects whose attendance at the exercise training sessions at the end of the intervention was below 75% were excluded from analysis.

VDR Genotype

High-molecular-weight genomic DNA was isolated from peripheral lymphocytes using standard techniques. Subjects were genotyped for the VDR FokI

and BsmI restriction sites as previously described (27,91), and grouped as FF, Ff, and ff, and BB, Bb, and bb genotypes, respectively.

Outcomes

Outcome measurements were made before the start of the aerobic exercise training intervention and were repeated at the end of the intervention. Subjects continued to exercise until all final measurements were made. All final measurements were made in a 24- to 36-hour time period following an exercise training session.

Plasma Glucose and Insulin Responses to an Oral Glucose Load

Subjects were instructed to consume at least 250 grams of carbohydrate per day for three days before the OGTT and to record all food consumed in a 3-day diet record. Additionally, a 12-hour overnight fast preceded the OGTT appointment. To minimize the possibility of a diurnal effect on results from the OGTT, all OGTT appointments were scheduled between 7:00 am and 9:00 am. On arrival for the OGTT appointment, subjects were questioned to confirm that they had complied with all preparation instructions. The diet record was collected and examined by GERS staff for a rough estimate of the adequacy of carbohydrate intake. Only if the subject's preparation was as described above was the OGTT performed.

A 20- or 22-gauge indwelling venous catheter was inserted into an upper limb vein at, or distal to, the antecubital fossa. Blood samples were drawn before and at 30, 60, 90, and 120 minutes after an oral 75 g dose of dextrose in a 296 mL (10 fluid ounce) solution. Samples were drawn into syringes and immediately transferred into tubes containing 15% EDTA to prevent coagulation, then stored on ice until further

processing. The catheter system was flushed and maintained between blood draws with 0.9% sodium chloride solution. All procedures were performed using aseptic technique. Glucose and insulin responses to the glucose load were calculated as total area under the response curve (AUC) using the trapezoidal method (2).

Insulin Sensitivity

Insulin sensitivity index (ISI) was calculated as described by Matsuda and DeFronzo (56) where ISI = 10,000/square root of [(fasting plasma glucose × fasting plasma insulin) × (mean OGTT glucose concentration × mean OGTT insulin concentration)]. ISI correlates well with euglycemic clamp measured insulin sensitivity values as reported by Matsuda and DeFronzo ($r = 0.73$, $p<0.0001$) (56), and Stumvoll et al. ($r = 0.66$, $p<0.0005$) (80), and with hyperglycemic clamp measured insulin sensitivity as reported by Chiu et al. ($r = 0.72$, $p<0.0001$) (19). Also, because the ISI measures are more feasible in terms of time, burden on the subjects, logistics, and costs, ISI can be measured on far more subjects than would be feasible if euglycemic clamp studies were used to assess insulin sensitivity.

Body Composition

Percent body fat was assessed using dual energy x-ray absorptiometry (DEXA) (model DPX-L or DPX-IQ, Lunar Corporation, Madison, Wisconsin) (57) scans of the whole body before and after the aerobic exercise training intervention. Intra-abdominal and subcutaneous adipose tissues (IAAT, SCAT) were assessed using computed tomography (CT). A 5-mm cross-sectional CT image of the abdomen in the region of lumbar vertebrae L4-L5 (120 kV, 2 second scan time) was recorded for each subject before and after the aerobic exercise training intervention

(GE High Speed Advantage, General Electric Medical Systems, Milwaukee, Wisconsin) (62).

Maximal Oxygen Uptake

All subjects underwent a maximal treadmill exercise test to assess maximal oxygen uptake (VO_2max) as an index of cardiovascular fitness. VO_2max was measured before the intervention to derive valid exercise prescriptions for the exercise training, and after the intervention to determine if a generalized cardiovascular training effect did in fact occur. The test started at 70% of the peak heart rate achieved on the subject's screening exercise test and treadmill grade was increased by 2% every two minutes. Blood pressure, heart rate, and electrocardiogram were monitored and the test was terminated when the subject could no longer continue. Thirty-second averages for oxygen consumption (VO_2) and respiratory exchange ratio (RER) were determined during the test using an online indirect calorimetry system composed of a breathing valve (model 2300, Hans Rudolph, Inc., Kansas City, Missouri), mixing chamber (Rayfield Equipment, Waitsfield, Vermont), medical gas analyzer (model 1100, Perkin Elmer, Inc., Danbury, Connecticut), and ventilation measurement module (VMM-400, Interface Associates, Aliso Viejo, California) interfaced to a microcomputer for online calculations using custom software. The highest VO_2 achieved during the test was designated VO_2max.

Sample Analyses

Plasma and serum were isolated from OGTT whole blood samples via centrifugation at 4°C and a relative centrifugal force of 1800 xg (3000 rpm, IEC 243

rotor) for 20 minutes. Supernatant plasma from each potassium EDTA containing tube was transferred to separate microcentrifuge tubes for glucose and insulin assays and stored at –80°C for later analyses. Samples collected before and after training on each subject were analyzed within a single assay to eliminate inter-assay variability as a confounding factor for training effects.

Plasma Glucose

Plasma glucose concentration was determined using the glucose oxidase method and a semi-automated glucose analyzer (2300 STAT PLUS, YSI Inc., Yellow Springs, Ohio). All samples were measured in duplicate. When duplicate assessments of a sample were discrepant by more than 2 mg/dL, the sample was re-assayed until two values differed by 2 mg/dL or less. All values within 2 mg/dL of each other were averaged to represent the glucose concentration for that sample.

Plasma Insulin

Immunoreactive plasma insulin concentration was determined via competitive radioimmunoassay (HI-14K kit, Linco Research, Inc., St. Charles, Missouri). Samples were analyzed in duplicate. When the duplicate values had a coefficient of variation of 10% or less, the average of the two values was used as the insulin concentration for that sample. Values for duplicates with coefficient of variation greater than 10% were re-evaluated in a subsequent assay and all values within one standard deviation of the mean of the four values were averaged to represent the insulin concentration for that sample.

Statistical Analyses

All statistical analyses were performed using SPSS 11.0 software (SPSS Inc., Chicago, Illinois). Data are presented as mean±standard error (SE), except for frequency data. Data for OGTT variables are presented as adjusted mean±SE. To address study hypotheses, the data were analyzed using two genotype groups. However, as sample sizes were sufficient, analyses were also performed with three genotype groups. Before statistical analyses were performed, variables violating homogeneity of variance and normal distribution assumptions were transformed. Specifically, ISI values at baseline were log transformed when analyzed both as two genotype groups and as three genotype groups, and change in insulin AUC values with training were squared when analyzed as two genotype groups. Gender, ethnicity, hormone replacement therapy (HRT) use, and Hardy-Weinberg equilibrium frequency differences were assessed using chi-square analysis. Except for OGTT variables, initial subject characteristics and training-induced change in variables were compared among genotype groups using analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used to compare initial values and training-induced change in OGTT variables among genotype groups. Baseline values were covaried for age, gender, ethnicity, HRT and intra-abdominal adipose tissue (IAAT). Change values were covaried for age, gender, ethnicity, HRT, initial value of the OGTT variable in question, and change in IAAT with training. HRT was utilized as a covariate based on evidence that HRT use may influence glucose metabolism, insulin sensitivity, and T2DM risk (24,44,51,73,90). Adjustment for IAAT was made due to the close association between IAAT and insulin sensitivity (74) and the effect of

abdominal obesity on increased risk of T2DM (1,46,58). Paired samples t-tests were used to analyze within group changes with training based on unadjusted values. Statistical significance was set at $p \leq 0.05$.

CHAPTER 3: RESULTS

VDR BsmI allele and genotype frequencies for the initial 119 subjects and for the 76 exercise training intervention subjects (Table 1) differed from previous reports (53,64,79,86), with the present study having more BB homozygotes and fewer Bb heterozygotes. Additionally, allele and genotype frequencies for both the initial study sample and for the exercise trained sample differed significantly from Hardy-Weinberg expectancies ($\chi^2 = 9.14$, $p = 0.003$ and $\chi^2 = 7.56$, $p = 0.006$, respectively). In an attempt to genotype the BsmI polymorphism using primers previously described (61), Ye et al. (87) observed that frequently in heterozygous subjects, the b allele containing the BsmI restriction site was poorly amplified. In testing their hypothesis that the allele drop-out by non-amplification resulted from a mismatch of a primer and its complementary binding site due to a polymorphism in the binding site, the Tru9I polymorphism in intron 8 of the VDR gene was identified. This A/G polymorphism is located in the reverse primer binding site of the primers originally used to genotype the BsmI polymorphism and the presence of the Tru9I A allele can result in allele drop-out when the BsmI polymorphism is genotyped with the original set of primers. Under some PCR and digestion conditions, subjects who are heterozygous for the BsmI polymorphism who also carry the Tru9I A allele can be mistaken for homozygous carriers of the BsmI only amplified allele. This finding was confirmed by Zajickova et al. (88). Using the original set of primers to genotype the BsmI polymorphism, Zajickova et al. found a higher number of BB genotypes with a relative lack of Bb heterozygotes. The genotype frequencies, BB 0.30, Bb 0.37, and bb 0.33, were very similar to those of the present study (Table 1), and as in

the present study, the genotype frequencies were not in Hardy-Weinberg equilibrium. It was found that the Tru9I polymorphism under the reverse primer binding site did lead to a drop out of the b allele in heterozygotes during PCR amplification, and thus to the false prevalence of BB genotypes. Based on this evidence, the primers used to genotype the BsmI polymorphism in the present study were examined and were found to match the original set of primers that result in the BsmI mis-genotyping, explaining the difference in allele and genotype frequencies from those previously reported and the deviation from Hardy-Weinberg equilibrium found in the present study. Due to this genotyping error, no further results for the BsmI polymorphism are reported.

Table 1. VDR BsmI gene polymorphism allele and genotype frequencies

	Allele		Genotype Frequency			χ^2
Initial	B	b	BB	Bb	bb	$\chi^2 = 9.14$
Study Sample	0.49	0.51	0.31	0.36	0.33	
	(117)	(121)	(37)	(43)	(39)	p = .003
Exercise-Trained	B	b	BB	Bb	bb	$\chi^2 = 7.56$
Sample	0.49	0.51	0.32	0.34	0.34	
	(74)	(78)	(24)	(26)	(26)	p = .006

Data are frequencies (n).

VDR FokI allele and genotype frequencies for the initial 120 subjects and for the 77 exercise training intervention subjects (Table 2) were similar to previous reports (20,53) and were in Hardy-Weinberg equilibrium ($\chi^2 = 0.02$, p = 0.88 and $\chi^2 = 0.65$, p = 0.42, respectively).

Table 2. VDR FokI gene polymorphism allele and genotype frequencies

	Allele		Genotype Frequency			χ^2
Initial	F	f	FF	Ff	ff	$\chi^2 = .02$
Study Sample	0.61	0.39	0.37	0.48	0.15	
	(146)	(94)	(44)	(58)	(18)	p = .88
Exercise-Trained	F	f	FF	Ff	ff	$\chi^2 = .65$
Sample	0.61	0.39	0.35	0.52	0.13	
	(94)	(60)	(27)	(40)	(10)	p = .42

Data are frequencies (n).

There were no significant differences in demographics, body composition, VO_2max, fasting plasma glucose (FPG), fasting plasma insulin (FPI), glucose area under the response curve (AUC), insulin AUC, or insulin sensitivity index (ISI) between the FokI genotype groups at baseline, whether analyzed as two (Table 3) or three groups (Table 4). However, when analyzed as two genotype groups, there was a tendency for FPI to differ (p = 0.091, covariates age, gender, ethnicity, HRT, IAAT), with FF homozygotes exhibiting higher FPI than f allele carries at baseline (Table 3).

Table 3. Initial subject characteristics

Characteristics	Total Group	VDR FokI Genotype Group		
		FF	Ff+ff	p
Age (yrs)	57.9±0.5	56.7±0.8	58.7±0.7	.064
Female/Male	73/47	28/16	45/31	.632
White/Non-White	83/37	28/16	55/21	.318
Females on HRT	32/73 (44%)	12/28 (43%)	20/45 (44%)	.894
Height (cm)	168.2±0.9	167.8±1.4	168.5±1.1	.701
Weight (kg)	82.1±1.3	83.0±2.3	81.6±1.6	.602
Body fat (%)	37.3±0.9	37.4±1.5	37.3±1.2	.940
IAAT (cm^2)	122.1±4.2	121.7±6.2	122.3±5.7	.944
SCAT (cm^2)	341.2±13.1	331.0±22.5	347.1±16.1	.555
VO_2max (L/min)	2.0±0.0	2.0±0.1	2.0±0.1	.996
VO_2max (mL/kg/min)	24.3±0.4	23.9±0.7	24.5±0.6	.502
FPG (mg/dL)	92.9±1.0	93.6±1.7	92.5±1.3	.593
FPI (pM/L)	84.3±2.8	90.7±4.7	80.6±3.6	.091
Glucose AUC	16219±321	16248±538	16203±409	.948
Insulin AUC	56142±2163	56850±3609	55728±2751	.806
ISI	3.5±0.2	3.3±0.3	3.6±0.2	.271

Values are expressed as mean±SE except for frequency data. Values for OGTT variables are expressed as adjusted mean±SE, adjusted for age, gender, ethnicity, HRT, and IAAT. ISI values by genotype group are reported untransformed. Log transformed values for ISI are FF 0.5±0.0, Ff+ff 0.5±0.0, p = .300. Sample size: Total Group = 106-120; FF = 39-44; Ff+ff = 67-76. p = statistical probability for the difference between genotype groups. IAAT = intra-abdominal adipose tissue; SCAT = subcutaneous adipose tissue; FPG = fasting plasma glucose; FPI = fasting plasma insulin; Glucose AUC measured in mg/dL x min; Insulin AUC measured in pM/L x min; ISI calculated according to the method of Matsuda and DeFronzo (56).

Table 4. Initial subject characteristics

		VDR FokI Genotype Group			
Characteristics	Total Group	FF	Ff	ff	p
Age (yrs)	57.9±0.5	56.7±0.8	58.5±0.8	59.2±1.4	.163
Female/Male	73/47	28/16	33/25	12/6	.677
White/Non-White	83/37	28/16	42/16	13/5	.608
Females on HRT	32/73 (44%)	12/28 (43%)	13/33 (39%)	7/12 (58%)	.522
Height (cm)	168.2±0.9	167.8±1.4	168.6±1.3	168.0±2.1	.905
Weight (kg)	82.1±1.3	83.0±2.3	83.0±1.9	76.9±2.7	.260
Body fat (%)	37.3±0.9	37.4±1.5	37.4±1.3	36.7±2.5	.963
IAAT (cm^2)	122.1±4.2	121.7±6.2	126.5±6.8	107.0±7.6	.327
SCAT (cm^2)	341.2±13.1	331.0±22.5	351.4±18.7	331.3±31.5	.743
VO_2max (L/min)	2.0±0.0	2.0±0.1	2.0±0.1	1.9±0.1	.772
VO_2max (mL/kg/min)	24.3±0.4	23.9±0.7	24.4±0.6	25.1±1.1	.683
FPG (mg/dL)	92.9±1.0	93.6±1.7	92.7±1.5	91.5±2.9	.815
FPI (pM/L)	84.3±2.8	90.7±4.7	80.1±4.0	82.4±7.8	.234
Glucose AUC	16219±321	16246±540	16120±462	16522±909	.924
Insulin AUC	56142±2163	56858±3625	56154±3117	54146±6030	.929
ISI	3.5±0.2	3.3±0.3	3.7±0.2	3.3±0.4	.362

Values are expressed as mean±SE except for frequency data. Values for OGTT variables are expressed as adjusted mean±SE, adjusted for age, gender, ethnicity, HRT, and IAAT. ISI values by genotype group are reported untransformed. Log transformed values for ISI are FF 0.5±0.0, Ff 0.5±0.0, ff 0.5±0.1, p = .535. Sample size: Total Group = 106-120; FF = 39-44; Ff = 53-58; ff = 14-18. p = statistical probability for the difference between genotype groups. IAAT = intra-abdominal adipose tissue; SCAT = subcutaneous adipose tissue; FPG = fasting plasma glucose; FPI = fasting plasma insulin; Glucose AUC measured in mg/dL x min; Insulin AUC measured in pM/L x min; ISI calculated according to the method of Matsuda and DeFronzo (56).

For the subjects who completed exercise training, age, gender, the proportion of white to non-white subjects, and hormone replacement therapy (HRT) use did not differ between the FokI genotype groups, whether analyzed as two (Table 5) or three groups (Table 6). Baseline values for body weight, percent body fat, intra-abdominal adipose tissue (IAAT), subcutaneous adipose tissue (SCAT), and VO_2max were

similar between the genotype groups when analyzed as two groups (Table 5) and as three groups (Table 6), as were baseline glucose AUC and insulin AUC (Tables 7 and 8). However, when grouped as FF homozygotes and f allele carriers, baseline FPG and FPI for subjects who completed exercise training differed significantly between the two groups, with FF homozygotes exhibiting higher FPG ($p = 0.038$, covariates age, gender, ethnicity, HRT, IAAT) and higher FPI ($p = 0.044$, covariates age, gender, ethnicity, HRT, IAAT) than f allele carriers. There was also a tendency for baseline ISI to differ between the two genotype groups among subjects who completed exercise training ($p = 0.097$, covariates age, gender, ethnicity, HRT, IAAT), with FF homozygotes exhibiting a lower ISI than f allele carriers (Table 7). There were no significant differences, though, in baseline FPG, FPI, or ISI between genotype groups among subjects who completed exercise training when analyzed as three groups (Table 8).

Table 5. Characteristics and changes with exercise among subjects who completed training

Characteristics	Total Group	VDR FokI Genotype Group		
		FF	Ff+ff	p
Age (yrs)	58.1±0.7	57.6±1.0	58.4±0.8	.568
Female/Male	44/33	15/12	29/21	.836
White/Non-White	56/21	19/8	37/13	.733
Females on HRT	17/44 (39%)	4/15 (27%)	13/29 (45%)	.241
Weight (kg)				
Baseline	80.1±1.6	81.9±3.0	79.2±2.0	.434
Change	-1.3±0.3*	-1.2±0.3*	-1.3±0.4*	.881
Body fat (%)				
Baseline	35.5±1.2	35.6±1.9	35.5±1.5	.985
Change	-1.3±0.2*	-0.9±0.3*	-1.5±0.3*	.210
IAAT (cm^2)				
Baseline	117.5±4.5	122.2±6.6	114.9±5.9	.440
Change	-10.8±2.5*	-9.7±3.4*	-11.5±3.5*	.734
SCAT (cm^2)				
Baseline	308.5±14.8	302.9±27.0	311.4±17.7	.788
Change	-6.5±4.7	-2.9±6.8	-8.5±6.2	.567
VO_2max (L/min)				
Baseline	2.0±0.1	2.1±0.1	2.0±0.1	.530
Change	0.3±0.0*	0.3±0.0*	0.3±0.0*	.219
VO_2max (mL/kg/min)				
Baseline	25.2±0.5	25.2±0.9	25.3±0.7	.938
Change	4.3±0.3*	4.7±0.6*	4.0±0.4*	.318

Values are expressed as mean±SE except for frequency data. Sample size: Total Group = 72-77; FF = 25-27; Ff+ff = 47-50. p = statistical probability for the difference between genotype groups. *Indicates significant change within group with training $p<.01$. IAAT = intra-abdominal adipose tissue; SCAT = subcutaneous adipose tissue.

Table 6. Characteristics and changes with exercise among subjects who completed training

		VDR FokI Genotype Group			
Characteristics	Total Group	FF	Ff	ff	p
Age (yrs)	58.1±0.7	57.6±1.0	58.4±0.9	58.5±2.0	.848
Female/Male	44/33	15/12	23/17	6/4	.969
White/Non-White	56/21	19/8	28/12	9/1	.421
Females on HRT	17/44 (39%)	4/15 (27%)	11/23 (48%)	2/6 (33%)	.407
Weight (kg)					
Baseline	80.1±1.6	81.9±3.0	79.7±2.3	77.1±3.7	.650
Change	-1.3±0.3*	-1.2±0.3*	-1.3±0.4*	-1.1±0.5*	.958
Body fat (%)					
Baseline	35.5±1.2	35.6±1.9	35.9±1.7	34.0±3.4	.870
Change	-1.3±0.2*	-0.9±0.3*	-1.5±0.3*	-1.7±0.6*	.441
IAAT (cm^2)					
Baseline	117.5±4.5	122.2±6.6	117.4±7.2	105.2±7.4	.505
Change	-10.8±2.5*	-9.7±3.4*	-14.0±3.9*	-2.0±7.5	.291
SCAT (cm^2)					
Baseline	308.5±14.8	302.9±27.0	314.5±19.8	299.4±41.8	.914
Change	-6.5±4.7	-2.9±6.8	-6.3±7.2	-16.6±13.0	.657
VO_2max (L/min)					
Baseline	2.0±0.1	2.1±0.1	2.0±0.1	2.0±0.1	.822
Change	0.3±0.0*	0.3±0.0*	0.3±0.0*	0.2±0.1*	.377
VO_2max (mL/kg/min)					
Baseline	25.2±0.5	25.2±0.9	25.1±0.8	26.0±1.0	.871
Change	4.3±0.3*	4.7±0.6*	4.1±0.4*	3.7±0.9*	.561

Values are expressed as mean±SE except for frequency data. Sample size: Total Group = 72-77; FF = 25-27; Ff = 37-40; ff = 9-10. p = statistical probability for the difference between genotype groups. *Indicates significant change within group with training $p \leq .05$. IAAT = intra-abdominal adipose tissue; SCAT = subcutaneous adipose tissue.

Table 7. OGTT baseline results and changes with exercise among subjects who completed training

		VDR FokI Genotype Group		
Characteristics	Total Group	FF	Ff+ff	p
FPG (mg/dL)				
Baseline	91.7±1.0	94.6±1.7	90.2±1.2	.038
Change	1.3±1.0	3.0±1.8	0.4±1.2	.234
FPI (pM/L)				
Baseline	82.0±3.0	90.5±5.1	77.5±3.7	.044
Change	-11.8±1.8*	-12.2±3.2*	-11.6±2.2*	.879
Glucose AUC				
Baseline	15925±358	16273±616	15740±447	.489
Change	-279±323	-113±583	-360±402	.733
Insulin AUC				
Baseline	57518±2663	55800±4597	58430±3332	.647
Change	-12232 ±1473*	-11925 ±2657*	-12382 ±1835*	.890
ISI				
Baseline	3.6±0.2	3.2±0.3	3.8±0.2	.097
Change	0.6±0.1*	0.5±0.2*	0.7±0.2*	.492

Values are expressed as adjusted mean±SE. Baseline values are covaried for age, gender, ethnicity, HRT, and IAAT. Change values are covaried for age, gender, ethnicity, HRT, initial value of the OGTT variable in question, and change in IAAT. Insulin AUC values by genotype are reported untransformed. Square transformed values for Insulin AUC are FF $2.58X10^{8}$±$1.15X10^{8}$, Ff+ff $4.39X10^{8}$±$7.92X10^{7}$, p = .205. Sample size: Total Group = 70-75; FF = 23-26; Ff+ff = 47-49. p = statistical probability for the difference between genotype groups. *Indicates significant change within group with training based on unadjusted values p<.007. FPG = fasting plasma glucose; FPI = fasting plasma insulin; Glucose AUC measured in mg/dL x min; Insulin AUC measured in pM/L x min; ISI calculated according to the method of Matsuda and DeFronzo (56).

Table 8. OGTT baseline results and changes with exercise among subjects who completed training

		VDR FokI Genotype Group			
Characteristics	Total Group	FF	Ff	ff	p
FPG (mg/dL)					
Baseline	91.7±1.0	94.6±1.7	90.0±1.4	90.6±2.8	.115
Change	1.3±1.0	3.0±1.8	0.1±1.4	1.3±2.7	.458
FPI (pM/L)					
Baseline	82.0±3.0	90.6±5.2	77.0±4.2	79.5±8.3	.130
Change	-11.8±1.8*	-12.3±3.2*	-11.1±2.6*	-13.5±4.9	.899
Glucose AUC					
Baseline	15925±358	16279±616	15496±503	16677±996	.456
Change	-279±323	-81±580	-649±459	637±870	.415
Insulin AUC					
Baseline	57518±2663	55795±4631	58606±3784	57756±7496	.897
Change	-12232 ±1473*	-11914 ±2681*	-12481 ±2119*	-12038 ±4023	.986
ISI					
Baseline	3.6±0.2	3.2±0.3	3.9±0.3	3.4±0.5	.162
Change	0.6±0.1*	0.5±0.2*	0.6±0.2*	0.8±0.4*	.700

Values are expressed as adjusted mean±SE. Baseline values are covaried for age, gender, ethnicity, HRT, and IAAT. Change values are covaried for age, gender, ethnicity, HRT, initial value of the OGTT variable in question, and change in IAAT. Sample size: Total Group = 70-75; FF = 23-26; Ff = 37-39; ff = 10. p = statistical probability for the difference between genotype groups. *Indicates significant change within group with training based on unadjusted values $p \leq .05$. FPG = fasting plasma glucose; FPI = fasting plasma insulin; Glucose AUC measured in mg/dL x min; Insulin AUC measured in pM/L x min; ISI calculated according to the method of Matsuda and DeFronzo (56).

With exercise training, when analyzed as two groups, body weight, percent body fat, and IAAT decreased significantly in both FF homozygotes (all $p<0.01$) and f allele carriers (all $p \leq 0.002$), while VO_2max increased significantly in both groups (all $p<0.001$). SCAT did not change significantly with training within either of the genotype groups (Table 5). In terms of change in OGTT variables with training, both

FF homozygotes and f allele carriers significantly decreased FPI (all $p \leq 0.006$) and insulin AUC (all $p < 0.001$) and significantly increased ISI (all $p \leq 0.004$). However, neither FPG nor glucose AUC changed significantly with training within either genotype group (Table 7). Finally, there were no significant differences between FF homozygotes and f allele carriers in training-induced changes for any of the outcome variables (Tables 5 and 7).

When analyzed as three groups, the FF, Ff, and ff genotype groups all significantly decreased body weight (all $p \leq 0.05$) and percent body fat (all $p \leq 0.002$) and significantly increased VO_2max (all $p \leq 0.003$) with training (Table 6). The FF and Ff genotype groups also significantly decreased IAAT (all $p \leq 0.009$) with training; however, the ff genotype group did not. SCAT did not change significantly with training within any of the genotype groups (Table 6). For the OGTT variables, the FF, Ff, and ff genotype groups all significantly increased ISI with training ($p = 0.004$, $p = 0.02$, and $p = 0.044$, respectively); however, only the FF and Ff genotype groups significantly decreased FPI ($p < 0.001$ and $p = 0.027$, respectively) and insulin AUC (all $p < 0.001$) with training, while the ff genotype group did not. FPG and glucose AUC did not change significantly with training within any of the genotype groups (Table 8). Finally, there were no significant differences between the FF, Ff, or ff genotype groups in training-induced changes for any of the outcome variables (Tables 6 and 8).

CHAPTER 4: DISCUSSION

Vitamin D and the vitamin D receptor (VDR) appear to play an important role in pancreatic beta cell function and glucose homeostasis. Vitamin D-deficient rats exhibit decreased de novo insulin synthesis (15), decreased pancreatic insulin content (48), decreased insulin secretion (17,48,63), and impaired glucose tolerance (17,18,89), while vitamin D administration activates pancreatic preproinsulin messenger RNA (mRNA) (68), restores insulin biosynthetic capacity (15), increases pancreatic insulin content (48), increases insulin secretion (18,48), and restores impaired glucose tolerance in vitamin D-deficient rats (18), and vitamin D-replete rats exhibit improved glucose clearance (17). In humans, individuals at risk for type 2 diabetes mellitus (T2DM) have significantly lower vitamin D levels (14), and vitamin D deficiency has been associated with insulin resistance (8), while vitamin D supplementation has been associated with improved insulin secretion in type 2 diabetics (12,39). Additionally, mice with nonfunctioning VDR exhibit impaired insulin secretion and oral glucose tolerance (89). The VDR gene has been examined as a candidate gene in the development of T2DM and the FokI and BsmI polymorphisms of the VDR gene have been associated with insulin sensitivity (20,26). Insulin sensitivity is determined by genetic and environmental factors (10) and exercise training improves insulin sensitivity (1,28). Therefore, the purpose of the present study was to evaluate the association of the FokI and BsmI polymorphisms of the VDR gene with changes in insulin sensitivity as a result of aerobic exercise training. Due to mis-genotyping of the BsmI polymorphism, results for that variant could not be reported. This outcome highlights the importance of

interpreting with caution the results of previous genetic association studies involving the BsmI polymorphism and not only glucose and insulin metabolism, but also other phenotypes that have been investigated in association with the BsmI variant, such as bone mineral density, especially when the allele and genotype frequencies differ from previous reports and/or deviate from Hardy-Weinberg equilibrium, as occurred in the present study.

For the FokI polymorphism, contrary to the study hypotheses, no significant association was found between the FokI polymorphism and insulin sensitivity either in the initial study sample before aerobic exercise training or with change in insulin sensitivity after aerobic exercise training in men and women at increased risk for T2DM. Additionally, there were no significant associations between the FokI polymorphism and any other variables derived from an oral glucose tolerance test (OGTT), including fasting plasma glucose (FPG), fasting plasma insulin (FPI), glucose area under the response curve (AUC), and insulin AUC, either in the initial study sample before exercise training or with change after exercise training, although baseline FPI in the initial study sample did tend to differ when analyzed as two genotype groups, with FF homozygotes tending toward higher FPI than f allele carriers. However, at baseline among subjects who completed exercise training, there were significant differences in FPG and FPI between FF homozygotes and f allele carriers, with FF homozygotes exhibiting higher FPG and FPI. As might be anticipated based on the higher FPG and FPI levels in FF homozygotes, insulin sensitivity index (ISI) also tended to differ at baseline among subjects who completed exercise training when analyzed as two genotype groups, with FF homozygotes

tending toward lower insulin sensitivity than f allele carriers. This tendency was opposite to the study hypothesis that before training, f allele carriers would have lower insulin sensitivity than FF homozygotes. The clinical significance of the difference in FPG between FF homozygotes and f allele carriers is unclear, though, as the magnitude of the difference is not great and with normoglycemia defined as FPG<110 mg/dL (5), both genotype groups were well within the normal range for FPG. There is, however, a larger difference in FPI values between the two genotype groups. FPI has been used as an indicator of insulin resistance (20); thus, the higher baseline FPI, along with the tendency toward lower baseline insulin sensitivity in FF homozygotes who completed exercise training, as well as the tendency for higher FPI in the initial sample of FF homozygotes, suggests that FF homozygotes are more insulin resistant than f allele carriers.

Chiu et al. (20) examined the role of the FokI polymorphism in glucose metabolism in a cross-section of men and women. FF homozygotes and f allele carriers were found to have similar plasma glucose levels during an OGTT; however, opposite to the results of the present study, f allele carriers had significantly higher insulin levels during the OGTT and significantly higher insulin AUC compared to FF homozygotes. Additionally, f allele carriers exhibited significantly lower insulin sensitivity than FF homozygotes. When analyzed as three genotype groups, the main difference in insulin sensitivity was between the FF and Ff genotype groups, while there was no difference between the Ff and ff genotype groups. In the present study, there were no significant differences in baseline insulin sensitivity when analyzed as three genotype groups. According to Zmuda et al. (92), conflicting results in genetic

association studies are to be expected and may occur for several reasons, including differences in ethnic (genetic) background, gene-gene and gene-environment interactions, and the definition of the phenotype. Chiu et al. (20) examined healthy, normotensive, normoglycemic Caucasian men and women at a mean age of 28 years (range 19-39 years) with a mean body mass index (BMI) of 24.3 kg/m^2 (range 17.6-34.3 kg/m^2). The physical activity level of the subjects was not assessed. Subjects in the present study were healthy, sedentary, nondiabetic, dyslipidemic and/or prehypertensive or hypertensive men and postmenopausal women at a mean age of 58 years (range 50-71 years) with a mean BMI of 29.0 kg/m^2 (range 19.8-40.2 kg/m^2) at baseline. Ethnic distribution in the present study was 69% Caucasian, 23% African-American, 3% Hispanic, and 5% Asian/Pacific Islander. Additionally, 44% of women in the present study were on hormone replacement therapy (HRT). Differences in the subjects examined may contribute to the conflicting results regarding the association of the FokI polymorphism with insulin values and insulin sensitivity between the two studies. Differences in the methods used may also account for the conflicting results. Chiu et al. (20) estimated insulin sensitivity using homeostasis model assessment (HOMA), while insulin sensitivity in the present study was estimated using the insulin sensitivity index (ISI) as described by Matsuda and DeFronzo (56).

There are a number of factors that may have contributed to the lack of further significant associations between the VDR FokI polymorphism and indices of glucose and insulin metabolism in the present study. Sample sizes may have been insufficient to detect genotype associations with the indices examined. According to Bergman et

al. (10), the validity of any measure of insulin sensitivity is particularly critical to its application to genetic studies, and the design of genetic studies must be based on accurate and precise indices that reveal the effect of insulin on tissue metabolic function. While it has been reported to correlate well with euglycemic and hyperglycemic clamp measured insulin sensitivity, ISI may not have been a sensitive enough measure of insulin sensitivity to detect differences between genotype groups. In an effort to reconcile the normal glucose and insulin responses to an OGTT found in children with resistance to $1,25(OH)_2D_3$, Hochberg et al. (34) speculated that although the OGTT results were normal, there may have been impairment of fine regulation of hormone release that could not be detected by a conventional test such as an OGTT. Alternatively, considering that the majority of research forming the basis for the investigation of the VDR gene in the etiology of T2DM demonstrates that vitamin D deficiency, vitamin D repletion, and dysfunction of the VDR are associated primarily with alterations in insulin secretion, more information may have been revealed by examining specific indices of insulin secretion in the present study.

According to Zmuda et al. (92), a major difficulty in accepting the hypothesis that known VDR allelic variants are directly responsible for observed associations is that none of the variants, with the possible exception of the FokI polymorphism, have consistently altered VDR expression or function in vitro. While the present study demonstrates an association of the FokI polymorphism with FPG and FPI, and a tendency for an association with insulin sensitivity, at baseline, Whitfield et al. (85) provide evidence that examination of the FokI polymorphism alone, despite its potential functionality, may not reveal significant effects. In an investigation of

endogenous VDR transcriptional activity in relation to VDR genotype at the unlinked FokI and poly(A) sites, a strong correlation was found between genotype and VDR activity when both polymorphic sites were considered simultaneously. The authors concluded that variation at both the FokI and poly(A) sites is important to VDR functional activity in vivo, but that considering each site separately may not reveal significant effects.

While further research is warranted, particularly in the areas of linkage disequilibrium and haplotype analysis (82) in an attempt to elucidate the possible role of the VDR gene in the etiology of T2DM, it may also be worthwhile to investigate other genes in an attempt to explain the observed link between vitamin D and VDR deficiency with glucose and insulin metabolism. To determine whether the stimulatory effect of vitamin D on insulin synthesis (89) and insulin secretion (41) is direct or indirect, Johnson et al. (41) and Zeitz et al. (89) have both suggested examining the insulin (INS) gene for a vitamin D response element (VDRE). Presence of VDRE in the insulin gene would suggest that the insulin gene may be a viable candidate for investigation. While there is no known report of VDRE in the insulin gene thus far, a VDRE has been identified in the insulin receptor (INSR) gene promoter. After demonstrating that $1,25(OH)_2D_3$ increases insulin receptor mRNA levels, insulin binding, and insulin responsiveness of U-937 human promonocytic cells, Maestro et al. (52) identified a sequence in the insulin receptor promoter that specifically binds VDR. The insulin receptor gene may be a viable candidate for study, as the cascade of signaling events that lead to insulin-stimulated glucose uptake is initiated by insulin binding to the insulin receptor (28). Also, while several

studies demonstrate that hypocalcemia during vitamin D deficiency is unlikely to be the cause of decreased insulin secretion and impaired glucose tolerance (17,42,89), the potential role of calcium in the association of vitamin D and VDR deficiency with glucose and insulin metabolism has not been completely ruled out. Ozono et al. (68) found in vivo activation of preproinsulin mRNA in the pancreas of vitamin D-deficient rats in response to calcium repletion, Ayesha et al. (6) found that decreased insulin secretion in vitamin D-deficient rats could be corrected by calcium supplementation, and in their examination of children with resistance to $1,25(OH)_2D_3$, Hochberg et al. (34) found that insulin secretion was subnormal only in subjects who were hypocalcemic. The major controlling factor in the stimulus-secretion coupling process of insulin release is an increase in intracellular calcium concentration in pancreatic beta cells (41,77). Calbindin D_{28k}, a calcium-binding protein whose expression is regulated by $1,25(OH)_2D_3$, is present in human and rat pancreatic islets (41). Treatment with $1,25(OH)_2D_3$ affects calbindin D_{28k} content of pancreatic islets (41) and it has been suggested that calbindin can control the rate of insulin release via regulation of cytosolic free calcium concentration (78). Thus, the gene encoding calbindin D_{28k} (CALB1) may be another possible candidate for study.

While there were no statistically significant genotype effects of the VDR FokI polymorphism in the initial study sample at baseline or for training-induced change for any of the outcome variables in the present study, the models for a number of indices of glucose and insulin metabolism were significant, including all models for FPG, baseline and change with training, when analyzed both as two and as three genotype groups. Interestingly, HRT was consistently a significant source of

variability in all of the models for FPG. HRT has been found to have an effect on glucose levels and T2DM risk in postmenopausal women. In the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial, after three years, women on HRT had lower fasting glucose levels, but higher 2-hour glucose levels, than women taking placebo (24). Likewise, in the Strong Heart Study (SHS), after an average of four years, American Indian postmenopausal women on HRT had lower fasting glucose but higher 2-hour glucose levels compared with women who had never used HRT. Also in the SHS, while HRT use was not significantly associated with the risk of T2DM compared with past and never HRT users, risk of T2DM did increase with increasing duration of estrogen use among current users (90). In the Heart and Estrogen/progestin Replacement Study (HERS), after approximately four years, fasting glucose levels increased significantly among postmenopausal women with coronary heart disease assigned to placebo but did not change among women receiving HRT, and the incidence of T2DM was 6.2% in the HRT group and 9.5% in the placebo group (44). The results of Rossi et al. (73) suggest that in healthy, nonobese postmenopausal women, the use of HRT reduces the risk of T2DM. After approximately four years, diabetes developed in 10% of HRT nonusers compared with 4.2% of HRT users and HRT nonusers had a relative risk of diabetes of 2.0 compared with HRT users. In the present study, sample sizes did not allow for separate genotype analyses of women on and not on HRT, but in examining fasting and 2-hour glucose levels in the initial study sample at baseline and for training-induced change, independent samples t-tests revealed that, consistent with previous findings, HRT users had lower FPG than HRT nonusers before training (87.7±1.8 vs.

95.6±1.9, respectively, p = 0.005). However, there was no difference in 2-hour glucose levels between HRT users and nonusers at baseline. With exercise training HRT users increased FPG levels, while HRT nonusers slightly decreased FPG (+5.4±2.3 vs. –1.5±1.6, respectively, p = 0.016). There was no significant difference in training-induced change in 2-hour glucose levels between HRT users and nonusers, though.

While HRT was not a significant source of variability in the models for FPI in the present study, there were also differences between HRT users and nonusers in insulin levels. FPI at baseline in the initial study sample and training-induced change in FPI tended to differ between HRT users and nonusers, with HRT users tending toward a lower FPI at baseline compared with HRT nonusers (75.3±5.9 vs. 88.4±4.5, respectively, p = 0.075). Both HRT users and nonusers decreased FPI with training; however, HRT nonusers tended to decrease FPI more than HRT users (-12.5±2.8 vs. –3.0±4.3, respectively, p = 0.063). While there was no difference between HRT users and nonusers in the initial study sample for 2-hour insulin levels, with training HRT nonusers decreased 2-hour insulin levels to a greater extent than HRT users (-136.5±27.0 vs. –28.1±39.9, respectively, p = 0.025). Consistent with the results of the present study, in the PEPI trial, women on HRT had lower fasting insulin levels than women taking placebo and there were no differences in 2-hour insulin levels between the two groups (24); however, in the SHS, there was no difference in fasting insulin levels between current and never or past users of HRT (90).

HRT was also not a significant source of variability in any of the models for ISI in the present study. However, HRT use has been shown to improve insulin

sensitivity (51), and in the present study, women on HRT tended to be more insulin sensitive than women not on HRT, with HRT users tending toward higher ISI values at baseline than HRT nonusers (4.1±0.4 vs. 3.2±0.3, respectively, $p = 0.058$). HRT status did not influence change in insulin sensitivity with training, though, as there was no difference in training-induced change in ISI between HRT users and nonusers. The possible influence of HRT use on glucose and insulin levels, insulin sensitivity, and training-induced change in these indices hinted at in the results of the present study, coupled with the results of previous studies, suggests that future investigations of the effects of HRT alone and in combination with exercise training on glucose and insulin metabolism are warranted.

In conclusion, while there were no significant associations of the VDR FokI polymorphism with baseline insulin sensitivity or other indices of glucose and insulin metabolism in the initial study sample, only a trend for baseline FPI to differ between FF homozygotes and f allele carriers, there were significant differences in baseline FPG and FPI, and a trend for baseline ISI to differ, between FF homozygotes and f allele carriers among subjects who completed exercise training. These results suggest that the FokI polymorphism may influence FPG and FPI levels, as well as the development of insulin resistance, in men and women at increased risk for T2DM. However, as there were no significant associations of the FokI polymorphism with changes in insulin sensitivity or any other outcome variables with exercise training, the FokI polymorphism does not appear to mediate training-induced changes in the indices of glucose and insulin metabolism examined in the present study. As the present study is the first known to examine the association of the FokI polymorphism

with changes in indices of glucose and insulin metabolism with aerobic exercise training, further research into the FokI polymorphism is warranted. Furthermore, it is important to analyze all known VDR polymorphisms and their interrelationships since they will interact with each other to determine VDR expression and activity (82). Also, as T2DM involves multiple interacting genes and environmental factors (9), it is important to explore other possible candidate genes and their interactive influence with exercise training to identify genes conferring an increased risk for T2DM and to determine the environmental factors that may attenuate the risk associated with those genes.

APPENDIX A: DELIMITATIONS

The present study was delimited as follows:

1. One hundred and six subjects were analyzed for baseline values of insulin sensitivity. Seventy subjects were analyzed for insulin sensitivity responses to aerobic exercise training. It is possible that these sample sizes were insufficient to detect genotype associations with insulin sensitivity.
2. All subjects who completed the Gene Exercise Research Study were 50-75 years of age, sedentary, non-diabetic, free from cardiovascular disease, and not taking medications known to affect glucose metabolism. Thus, the results of the present study most specifically apply to populations with similar characteristics.
3. All subjects who completed the Gene Exercise Research Study were recruited from College Park, Maryland and the surrounding areas; therefore, the results of the present study may not be representative of the entire population.
4. All women who completed the Gene Exercise Research Study were at least two years postmenopausal and may or may not have been on hormone replacement therapy (HRT). Since HRT may affect glucose and insulin metabolism, it is possible that HRT status affected genotype and/or training effects on insulin sensitivity.
5. Polymorphisms in the regions flanking the VDR FokI variant were not identified or assessed in the present study. It is therefore possible that the reported genotype effects of FokI are due to linkage disequilibrium between FokI and a distinct polymorphism.

APPENDIX B: LIMITATIONS

Results from the present study depend on the following limitations:

1. All subjects who completed the Gene Exercise Research Study self-reported many factors related to health and lifestyle such as physical activity habits, dietary habits, medication regimens, and medical conditions. It is possible that inaccurate self-reports affected the results of the present study.
2. The Gene Exercise Research Study does not include a control group. As such, it is possible that factors such as seasonal variation in outcome measures or subject aging influenced the results of the present study. Furthermore, as subjects were volunteers, they were a convenience sample, rather than a random sample.
3. Vitamin D status of subjects was not assessed in the present study. Since vitamin D level may affect insulin resistance and insulin secretion, it is possible that vitamin D status affected genotype and/or exercise training effects on insulin sensitivity.
4. Calcium status of subjects was not assessed in the present study. Since calcium level may affect insulin secretion, it is possible that calcium status affected genotype and/or exercise training effects on insulin sensitivity.
5. The competitive radioimmunoassay used for quantification of plasma insulin concentrations is specific for immunoreactive insulin. It is possible that other plasma constituents such as proinsulin and partially degraded insulin, which are immunoreactive but not biologically active, resulted in errors in estimation of biologically active insulin concentrations.

APPENDIX C: DEFINITION OF TERMS

Alopecia: The partial or complete absence of hair from areas of the body where it normally grows (40).

C-peptide: In the processing of proinsulin, the prohormone is cleaved into active insulin and an inactive fragment known as C-peptide. C-peptide levels are measured in the blood to monitor how much insulin an individual's pancreas is producing (77).

Charcot joints: A neuropathic joint disease resulting from nerve damage that impairs a person's ability to perceive pain coming from a joint. Consequently, repeated minor injuries and fractures go unnoticed until the accumulated damage permanently destroys the joint (11).

Diabetes mellitus: A group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Three ways to diagnose diabetes are possible, and each must be confirmed, on a subsequent day, by any of the three methods: 1) Symptoms of diabetes plus casual plasma glucose concentrations ≥200 mg/dL (11.1 mmol/L) or 2) fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) or 3) 2-hour postload glucose ≥200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (71).

Exercise: A subset of physical activity defined as planned, structured, and repetitive bodily movement done to improve or maintain one or more components of physical fitness (70).

Glucose toxicity: Insulin secretion is abnormal in T2DM and the concept that hyperglycemia alone may further inhibit insulin secretion has been termed glucose toxicity (1).

Heritability (h^2): The proportion of the variance of a phenotype that is attributed to additive effects of genes. Heritability is the primary measure of the strength of genetic determination (10).

Impaired fasting glucose/impaired glucose tolerance: Terms referring to a metabolic stage intermediate between normal glucose homeostasis and diabetes. Fasting plasma glucose ≥110 mg/dL (6.1 mmol/L) but <126 mg/dL (7.0 mmol/L) is termed impaired fasting glucose (IFG). The corresponding category when the oral glucose tolerance test is used is 2-hour postload glucose ≥140 mg/dL (7.8 mmol/L) and <200 mg/dL (11.1 mmol/L), termed impaired glucose tolerance (IGT). IFG and IGT are not clinical entities in their own right, but rather are risk factors for T2DM, at least in part because of their correlation with insulin resistance, and can be viewed as intermediate stages in the disease process (71).

Impaired insulin secretion: Type 2 diabetics may have normal or elevated insulin levels, but despite this, are hyperglycemic. The higher blood glucose levels would be expected to result in even higher insulin levels, but pancreatic beta cell function is not normal in T2DM and insulin secretion is defective and insufficient to compensate for the insulin resistance (71,77).

Insulin: The major hormone that promotes anabolism in the human body. Insulin promotes the cellular uptake of glucose and its conversion to glycogen and fat, as well as the cellular uptake of amino acids and their conversion to proteins. Insulin's primary targets are the liver, adipose tissue, and skeletal muscle (77,83).

Insulin resistance: The subnormal biologic response to a given concentration of insulin (30).

Insulin sensitivity: The degree of tissue responsiveness to insulin (83).

Ligand: The molecule that binds to a receptor (77).

Pancreatic beta cells: The pancreas is both an endocrine and an exocrine gland, with the endocrine portion consisting of scattered clusters of cells called the pancreatic islets, which contain alpha and beta cells. The beta cells produce and secrete insulin (83).

Physical activity: Any bodily movement produced by skeletal muscles that results in energy expenditure (70).

Proinsulin: Proinsulin is a prohormone, an inactive protein containing one or more copies of a hormone (77).

Transcription: Transfer of information coded in DNA to messenger RNA (77).

Transcription factor: A molecule that binds to DNA and turns on one or more genes (77).

Type 2 diabetes mellitus (T2DM): The most prevalent form of diabetes, caused by a combination of insulin resistance and an inadequate compensatory insulin secretory response (71).

Vitamin D (1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]): Vitamin D functions like a steroid hormone, and its principal physiologic function is to maintain intracellular and extracellular calcium and phosphorus concentrations within a physiologically acceptable range. $1,25(OH)_2D_3$, the biologically active form of vitamin D, interacts with a specific nuclear receptor in its target tissues that results in a biologic response. A variety of tissues that are not related to calcium metabolism, including the pancreas, possess nuclear receptors for $1,25(OH)_2D_3$ (29,36).

Vitamin D receptor (VDR): Vitamin D interacts with nuclear vitamin D receptor proteins (VDR) to influence gene transcription. Vitamin D binds to VDR, which initiates a conformational change that in turn increases the receptor's affinity to vitamin D response elements (VDRE). The VDRE are found in the promoter regions of specific target genes. Once the vitamin D-VDR complex is bound to the VDRE, transcription of genes for specific mRNA coding for proteins may be either enhanced or inhibited (29).

APPENDIX D: REVIEW OF LITERATURE

Public Health Importance of Diabetes Mellitus

Diabetes mellitus is an etiologically and clinically heterogeneous group of diseases that share hyperglycemia in common. This hyperglycemia results from defects in insulin secretion, insulin action, or both (71). Diabetes is one of the leading causes of death and disability in the United States (1), as the chronic hyperglycemia of the disease is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Specifically, the long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputation, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Diabetics have an increased incidence of atherosclerotic cardiovascular, peripheral vascular, and cerebrovascular disease, and hypertension, abnormalities of lipoprotein metabolism, and periodontal disease are also often found in diabetics (71).

As of 2002, an estimated 12.1 million people in the United States have been diagnosed with diabetes; however, the prevalence of the disease is believed to be much greater, as it has been estimated that as many as one-third of people with diabetes are unaware that they have the disease. Not only is diabetes a significant cause of morbidity and mortality, with an estimated 186,000 deaths attributed to the disease in 2002, it is also one of the most costly chronic diseases. An estimated $92 billion in health care expenditures and an estimated $40 billion in lost productivity due to disability and early mortality resulted in an estimated national cost of diabetes

of $132 billion in 2002. As the United States' population grows in size, ages, and becomes more racially and ethnically diverse, the size of the population diagnosed with diabetes will also grow. Due to the projected increase in the number of people diagnosed with diabetes to approximately 14.5 million by 2010 and 17.4 million by 2020, the annual cost in 2002 dollars of diabetes could rise to an estimated $156 billion by 2010 and to $192 billion by 2020, although the actual future cost of diabetes will likely be substantially higher than these estimates (4).

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM), which results from insulin resistance with an insulin secretory defect (71), is the most prevalent form of diabetes, accounting for 90-95% of all diagnosed cases of the disease (1). The risk of developing T2DM increases with age (≥45 years), obesity (body mass index ≥25 kg/m^2), and physical inactivity (5). T2DM is more common in individuals with a family history of the disease and in African-Americans, Hispanic-Americans, Native Americans, Asian-Americans, and Pacific Islanders (1,5). The disease occurs more frequently in women with a history of gestational diabetes mellitus, delivery of a baby weighing greater than nine pounds, or polycystic ovary syndrome. T2DM also occurs more frequently in individuals with hypertension (≥140/90 mm Hg in adults), dyslipidemia (HDL cholesterol ≤35 mg/dL and/or TG level ≥250 mg/dL), impaired fasting glucose (fasting plasma glucose ≥110 and <126 mg/dL), impaired glucose tolerance (2-hour postload glucose ≥140 and <200 mg/dL), or history of vascular disease. The greater the number of risk factors an individual has for T2DM, the more likely that individual is to develop the disease (5).

Pathophysiology of Type 2 Diabetes Mellitus

Hyperglycemia is the hallmark of T2DM (83) and is used to define the disease (16). There are three ways to diagnose T2DM, and each must be confirmed, on a subsequent day, by any of the three methods. T2DM can be diagnosed by fasting plasma glucose ≥126 mg/dL, by 2-hour postload glucose ≥200 mg/dL, or by symptoms of diabetes plus casual plasma glucose ≥200 mg/dL (5). While glucose levels are the diagnostic criteria used for T2DM, it is the pancreatic beta cell and the hormone insulin that are central in the pathophysiology of the disease (9).

Insulin, a peptide hormone produced and secreted by the pancreatic beta cells, is the major hormone that promotes anabolism in the human body, and is also the primary regulator of blood glucose concentration. The primary stimulus for insulin secretion is an increase in blood glucose concentration. Insulin acts to maintain normoglycemia by increasing glucose uptake in skeletal muscle, liver, and adipose tissue, and by inhibiting gluconeogenesis. Insulin also promotes the storage of glucose and amino acids in adipose tissue, liver and skeletal muscle by stimulating lipogenesis, glycogen and protein synthesis, and by inhibiting lipolysis, glycogenolysis and protein breakdown (75,77,83). Insulin resistance or deficiency results in profound dysregulation of these processes and produces the elevations in fasting and postprandial glucose levels (75) characteristic of the disease process of T2DM.

A common pathophysiological model for the development of T2DM includes several metabolically distinct stages, which develop over many years. An early stage is defined by normoglycemia in the presence of peripheral insulin resistance (58).

Insulin resistance is associated with hyperinsulinemia, which results from a combination of an increase in insulin secretion and a decrease in insulin clearance rates. Due to the resistance to insulin action, more insulin is secreted at each level of glucose; by how much more represents the extent of the pancreatic beta cell adaptation necessary to maintain normal glucose tolerance (9). Eventually, though, there is a progressive decrease in insulin secretion as glucose levels rise, either by unmasking a primary defect in insulin secretion, from pancreatic beta cell exhaustion, from glucose toxicity, or from a combination of these defects, and the pancreatic beta cell can no longer secrete sufficient insulin to maintain normal blood glucose levels (9,30). Thus, at a later stage in the disease process characterized by impaired glucose tolerance, while fasting glucose is still normal, post-prandial glucose escapes the control of circulating insulin, resulting in post-prandial hyperglycemia. At the final stage in the development of the disease, most individuals still have circulating insulin, but the quantities are insufficient to maintain even fasting normoglycemia (58). Thus, the cause of T2DM is both a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (71).

Vitamin D Receptor Gene and Polymorphisms

T2DM is a complex trait associated with genetic, environmental, and metabolic defects that contribute to the key defects of insulin resistance and impaired insulin secretion (58). The complex pathophysiology of T2DM suggests that many pathways, and therefore many genes, contribute to the disease (16). A gene that has been investigated as a candidate gene in the development of T2DM is the gene encoding the vitamin D receptor (VDR). The VDR is a member of the superfamily of

nuclear hormone receptors (31,45,85) and is a ligand-activated transcription factor that mediates the biological actions of vitamin D (45,85). The VDR gene is located at human chromosome 12q12-q14 and contains 14 exons. Exons IA through IF encode the 5' untranslated region, exons II and III encode the DNA-binding domain (92), and exons VI-IX encode the ligand-binding domain, with exon IX also containing the 3' untranslated region (85). Several common genetic variants have been identified in the VDR gene. Most of these variants, including the FokI, BsmI, Tru9I, EcoRV, ApaI, and TaqI polymorphisms, are identified by a biallelic variation in a restriction endonuclease site and are named based on the restriction endonuclease (82,85). Genotypes for these polymorphisms are designated by the first letter of the name of the enzyme, except in the case of the Tru9I polymorphism, which has been designated U/u to avoid confusion with the TaqI genotypes. A capital letter indicates the absence of the cut site, whereas a lower-case letter indicates its presence (87).

In the 5' region of the VDR gene, exon IE contains the Cdx2 polymorphism, a G/A polymorphism in a binding site for an intestinal-specific transcription factor, called Cdx2 (82). Exon 2 contains the FokI polymorphism, a T/C transition polymorphism (ATG to ACG) that occurs at the first of two potential translation initiation sites (92) and results in an alteration of the VDR protein structure (85). Individuals with the T allele (designated f, indicating the presence of the FokI site) express the M1 isoform of VDR, so named because it contains the ATG methionine translational start site corresponding to the first codon (31), and initiate translation at the first ATG site to synthesize the full-length (427 amino acid) VDR protein (92). Individuals with the C allele (designated F, indicating the absence of the FokI site)

express the M4 isoform, initiating translation at the second ATG site, which corresponds to the fourth codon. These individuals lack the three NH_2^- terminal amino acids of the full-length VDR protein, resulting in a protein of only 424 amino acids (31,85,92). Thus, Ff and ff genotypes express the full-length VDR isoform, while the FF genotype expresses the shorter isoform (92). The F alleles of the FokI polymorphism appear to be more transcriptionally active than the f alleles (31,85).

Genetic variation in the 3' region of the VDR gene includes the BsmI, Tru9I, EcoRV, and ApaI polymorphisms in intron 8 (82), a silent TaqI polymorphism in exon 9, and a singlet (A) repeat [poly(A)] in the portion of exon 9 encoding the 3' untranslated region (85). The BsmI and ApaI polymorphisms are unlikely to have functional consequences as these variants are located in an intron and neither variant is near the intron-exon boundaries or known to produce splicing errors. However, while similar VDR protein and messenger RNA (mRNA) levels, ligand-binding affinity, DNA binding, and transactivation function have been found between BsmI genotypes (92), it has also been reported that the b alleles appear to be more active than the B alleles (85). A T/C nucleotide substitution (ATT to ATC) leading to a silent mutation in exon 9 characterizes the VDR TaqI polymorphism (31,92). This polymorphism is also unlikely to directly affect VDR function, since both alleles code for isoleucine at amino acid 352 (92). The poly(A) variants are mononucleotide repeat polymorphisms classified as either 'long' (L) or 'short' (S) by the number of consecutive A's in the repeat (85,92). While the poly(A) variants are expressed in the mature mRNA for VDR (85), they do not appear to alter VDR mRNA stability. The 3' polymorphisms are in linkage disequilibrium, and as these polymorphisms do not

appear to alter VDR gene expression or VDR function, disease associations with these polymorphisms are most likely due to linkage disequilibrium with other functional variation within the VDR gene or with another closely linked gene or genes (92). On the other hand, the 5' FokI polymorphism is a potentially functional variant and it does not appear to be in linkage disequilibrium with the 3' polymorphisms (31,92).

Vitamin D and its Mechanism of Action through the Vitamin D Receptor

1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is the biologically active form of vitamin D and functions like a steroid hormone (29,36). The traditional action of vitamin D, via its 1,25$(OH)_2D_3$ hormonal metabolite, is to maintain serum calcium and phosphorus concentrations in a range that supports cellular processes, neuromuscular function, and bone ossification (31,36). The actions of vitamin D are mediated both by genomic and non-genomic pathways. The genomic pathways are activated by the binding of 1,25$(OH)_2D_3$ to nuclear VDR. The non-genomic pathways are activated via cell membrane receptors for vitamin D, and might be responsible for the rapid effects of the hormone (86). However, most of the biological actions of vitamin D are believed to be exerted through the nuclear VDR-mediated control of target genes (45).

1,25$(OH)_2D_3$ acts as a ligand for the VDR, which then activates target gene expression at the transcriptional level (45). Specifically, 1,25$(OH)_2D_3$ enters its target cell and binds with nuclear VDR. The 1,25$(OH)_2D_3$-VDR complex then binds with retinoid X receptor (RXR) to form a heterodimer that interacts with DNA sequences known as vitamin D response elements (VDRE). The VDRE are found in the

promoter regions of target genes, and once the heterodimeric complex is bound to the VDRE, transcription of genes for specific mRNA coding for proteins may be either enhanced or inhibited (29,31,36,45).

Evidence for the Vitamin D Receptor Gene as a Candidate Gene in the Development of Type 2 Diabetes Mellitus

A candidate gene has traditionally been defined as one whose dysfunction might reasonably be expected to cause a disease because of evidence that is not directly genetic, such as evidence from biochemical, cellular, clinical, physiological, or pathological experiments (16). While it is doubtful that dysfunction of the VDR gene alone would cause T2DM, as the disease is polygenic (75), the evidence for a possible role of the VDR gene in the etiology of the disease is based on the fact that vitamin D and its receptor complex appear to play an important role in the mechanism of action of insulin release and in the maintenance of glucose tolerance.

While vitamin D traditionally is known for its role in the maintenance of calcium and phosphorus homeostasis, the hormone has a number of functions in a wide variety of tissues and cells, both related to calcium metabolism and unrelated to calcium metabolism. One of those noncalcemic tissues is the pancreas (35). The VDR is present in pancreatic beta cells of humans and rats (41). Some of the most compelling evidence for a possible role of the VDR gene in the development of T2DM is based on the finding that mice with nonfunctioning VDR exhibit impaired glucose tolerance and reduced maximum insulin secretory capacity, demonstrating a molecular role of the VDR in insulin synthesis and secretion in vivo. Zeitz et al. (89) generated gene-targeted mice expressing functionally inactive mutant VDR. As calcium plays an important role in the regulation of insulin secretion in pancreatic

beta cells (41), mutant mice were fed a rescue diet, which normalized body weight and mineral homeostasis in VDR mutants compared to wild-type mice, to rule out possible influences of hypocalcemia on pancreatic endocrine function. In glucose tolerance tests, baseline blood glucose levels were unchanged in fasting VDR mutants; however, blood glucose was elevated after oral or subcutaneous glucose loading, and maximum serum insulin levels were reduced by ~60% in VDR mutants compared to wild-type mice. Additionally, insulin mRNA levels were decreased in VDR mutant mice, while pancreatic beta cell mass, islet architecture, and islet neogenesis were normal, indicating that the impaired glucose tolerance and insulin secretion in VDR mutant mice could not be explained by alterations in pancreatic beta cell mass or microanatomical changes within pancreatic islets.

The examination of mice with nonfunctioning VDR was conducted in an effort to further explore the functional role of vitamin D in the endocrine pancreas. Some of the first evidence that vitamin D may act directly at the cellular level to influence pancreatic beta cell function came from studies of vitamin D-deplete and -replete rats. Norman et al. (63) found that pancreases from vitamin D-deficient rats exhibited a 48% decrease in insulin secretion compared to pancreases from vitamin D-replete rats. In a subsequent study, Kadowaki and Norman (42) investigated whether the effects of vitamin D and its metabolites on insulin secretion from the perfused rat pancreas in vitro were direct in action or whether they were secondary to the hypocalcemia or reduced caloric and calcium intake associated with vitamin D deficiency. It was found that normalization of serum calcium in vitamin D-deficient rats did not normalize insulin secretion and that the equalization of dietary caloric

intake between vitamin D-deficient and vitamin D-replete rats did not eliminate the effect of vitamin D on mediating insulin secretion. Cade and Norman (17) then examined the influence of vitamin D status on insulin secretion in vivo by performing intravenous glucose tolerance tests (IVGTT) on vitamin D-deficient and vitamin D-replete rats. The vitamin D-deficient rats exhibited impaired glucose tolerance and a corresponding 50% decrease in insulin response. Vitamin D repletion significantly improved glucose clearance, while normalization of plasma calcium levels of the vitamin D-deficient rats failed to improve glucose clearance or insulin secretion. Cade and Norman (18) also examined the effect of a single dose of $1,25(OH)_2D_3$ on glucose tolerance and insulin secretion in vitamin D-deficient rats using an IVGTT and found that $1,25(OH)_2D_3$ restored both impaired glucose tolerance and decreased insulin secretion. Labriji-Mestaghanmi et al. (48) supplied further evidence that vitamin D deficiency causes alterations of pancreatic beta cell function in rats, finding that vitamin D deprivation decreased and vitamin D treatment increased the insulin content of the whole pancreas or isolated pancreatic islets and the secretory response of the islets to glucose. Additionally, Bourlon et al. (15) found that de novo insulin synthesis was reduced in isolated pancreatic islets from vitamin D-deficient rats and that addition of $1,25(OH)_2D_3$ in vitro restored insulin biosynthetic capacity and produced an acceleration of the conversion of proinsulin to insulin. However, Ayesha and Raghuramulu (7) found unaltered oral glucose tolerance in vitamin D-deficient rats and, while Ozono et al. (68) found in vivo activation of preproinsulin mRNA in the pancreas of vitamin D-deficient rats, this enhancement only occurred under conditions of refeeding and during feeding, but not during fasting. The activation of

preproinsulin mRNA also occurred in response to calcium repletion. Also, Ayesha et al. (6) found that decreased insulin secretion in vitamin D-deficient rats could be corrected by calcium supplementation and Lee et al. (49) found that $1,25(OH)_2D_3$ failed to stimulate insulin secretion in vitro in pancreatic islets from vitamin D-replete rats and insulinoma beta cells.

The influence of vitamin D on glucose and insulin metabolism has also been examined in humans. In East London Asians, Boucher et al. (14) found plasma vitamin D levels were significantly higher in healthy controls than in subjects at risk for T2DM. Baynes et al. (8) found an inverse relationship between one-hour glucose and glucose AUC during OGTT and vitamin D concentration in elderly Dutchmen, and, after excluding diabetics, an inverse relationship between total insulin concentrations during OGTT and vitamin D concentration. In type 2 diabetics, Inomata et al. (39) found that total insulin secretion in response to an OGTT was significantly increased with vitamin D supplementation and Borissova et al. (12) found in vitamin D-replete type 2 diabetics that the first phase of insulin secretion (FPIS) during an IVGTT was significantly increased with a significant correlation between the change in FPIS and the change in vitamin D level after supplementation. However, Orwoll et al. (67) did not find an effect of vitamin D treatment on fasting or stimulated glucose, insulin, or C-peptid concentrations in type 2 diabetics.

While a functional role of the VDR in the endocrine pancreas of mice has been demonstrated, the same has not been shown in humans thus far. Hochberg et al. (34) examined glucose and insulin responses to an OGTT in a small sample of children with the syndrome of resistance to $1,25(OH)_2D_3$ with rickets and alopecia.

The occurrence of the syndrome is due to defective binding or lack of receptors for $1,25(OH)_2D_3$. In the case of these children, the receptors for $1,25(OH)_2D_3$ were defective. Glucose levels were normal in all subjects and insulin secretion was normal in subjects studied while normocalcemic, but was subnormal in hypocalcemic subjects. The latter results suggest that hypocalcemia per se, and not lack of a direct effect of $1,25(OH)_2D_3$ on the pancreas, may be the cause of the impaired insulin secretion.

FokI and BsmI Polymorphisms of the Vitamin D Receptor Gene and Glucose and Insulin Metabolism

Despite the lack of a clear answer to the question of the influence of vitamin D and VDR deficiency on glucose tolerance and insulin secretory capacity in humans, genetic association studies have been conducted examining the role of the VDR gene in glucose and insulin metabolism. Chiu et al. (20) examined the role of the FokI polymorphism in healthy, normotensive, glucose tolerant Caucasians who underwent an OGTT. FF homozygotes and f allele carriers had similar plasma glucose levels during the OGTT; however, f allele carriers had significantly higher insulin levels at 30, 60, and 90 minutes during the OGTT and significantly higher insulin area under the response curve (AUC) than the FF genotype group. There was no significant difference in pancreatic beta cell function (%B) between the two groups, but insulin sensitivity (%S) was significantly different between the genotype groups, with f allele carriers exhibiting lower insulin sensitivity than FF homozygotes. The influence of genotypes on %B and %S was also examined among the three genotype groups. Again, there was no difference in %B while %S was significantly different. The main

difference in %S was between the FF and Ff genotype groups. There was no difference in %S between the Ff and ff genotype groups.

A number of investigators have also examined the role of the BsmI polymorphism in glucose and insulin metabolism. In an investigation of insulin secretion in nondiabetic Bangladeshi Asians, Hitman et al. (33) found no significant association between insulin secretion index or 32,33 split proinsulin (used as an estimate of insulin resistance) and the BsmI polymorphism. Oh et al. (64) evaluated the association between the BsmI polymorphism and insulin secretion and insulin resistance in older nondiabetic Caucasian men and women. While there was no significant association between genotype and insulin resistance after adjusting for age, sex, and body mass index, after additional adjustment for calcium and vitamin D use, insulin resistance was significantly higher in subjects with the bb genotype compared to those with the BB genotype. Gabel et al. (26) examined the relationship between the BsmI polymorphism and fasting plasma glucose (FPG), fasting plasma insulin (FPI), insulin sensitivity index (S_I), acute insulin response to glucose (AIR_g), and glucose tolerance (K_g) in healthy postmenopausal Caucasian women categorized as sedentary, physically active, or athletic. In the total group, there was no significant association between the BsmI polymorphism and FPG, FPI, S_I, AIR_g, or K_g. However, when adjusted for VO_2max, the polymorphism was significantly associated with S_I. The BB genotype group had a significantly greater S_I than the Bb genotype group, and there was a trend for the BB genotype group to have a greater S_I than the bb genotype group. Speer et al. (79) evaluated the relationship between the BsmI polymorphism and insulin secretion in Caucasians with T2DM or android obesity

with normal carbohydrate metabolism and normal glucose tolerance, compared with healthy controls. There were no differences in BsmI genotype distribution between the three groups, but in the diabetics and subjects with android obesity, one-hour postprandial C-peptide levels were significantly higher in subjects with the BB genotype compared to subjects with the Bb genotype, suggesting that these genotypes could be predisposing factors that might contribute to pathogenetic processes leading to T2DM in android obesity. In a study designed to test the influence of the BsmI polymorphism on the prevalence of T2DM among subjects at high risk for coronary artery disease, Ortlepp et al. (65) found the BB genotype had the highest prevalence of T2DM and had an odds ratio of 3.6 for T2DM compared to the bb genotype, suggesting that individuals with the BB genotype have a higher risk of developing T2DM. However, Ye et al. (86) did not find an association between T2DM and the BsmI polymorphism in Caucasian diabetics.

Physical Activity and Type 2 Diabetes Mellitus

Currently, little is known about the specific genetic susceptibility variants that contribute to T2DM (58) because the genetic basis of the disease is complex, involving both multiple interacting genes and environmental factors, which determine whether diabetes will develop (9). Thus, the genetic determinants likely do not act as direct determinants of the disease, but instead determine susceptibility within the prevailing environmental context (16). One of the environmental factors involved in the development of T2DM is physical inactivity. The classification of habitual physical inactivity as a risk factor for T2DM is supported by a number of studies that have investigated the association between level of physical activity and incidence of

T2DM. Helmrich et al. (32) found that leisure-time physical activity was inversely related to the development of T2DM in men, and that vigorous activity was the most effective in decreasing risk for development of the disease. The protective effect of physical activity was also found to be greatest in those men at the highest risk for the disease. Similarly, Manson et al. (54) found that regular vigorous exercise was associated with a decreased incidence of T2DM in men and that the benefit also appeared to be greatest in those men at highest risk for the disease. Manson et al. (55) also examined the association between physical activity and subsequent incidence of T2DM in women and found a reduced incidence of the disease among women who exercised regularly compared with their sedentary counterparts. However, unlike the results in men, the benefits were evident to a similar extent in both women at high and at low risk for the disease.

Tuomilehto et al. (81) examined the prevention of T2DM by changes in lifestyle among subjects with impaired glucose tolerance. In the intervention group, which received individualized counseling aimed at reducing weight, total intake of fat, and intake of saturated fat and increasing fiber intake and physical activity, the risk of T2DM was reduced by 58% compared to the control group. The Diabetes Prevention Program Research Group (22) also utilized lifestyle intervention, as well as pharmacological intervention, to examine the reduction in the incidence of T2DM in nondiabetic men and women with elevated fasting and post-load plasma glucose concentrations. As compared with placebo, the lifestyle intervention, which aimed to achieve and maintain a weight reduction of at least 7% of initial body weight through a healthy low-calorie, low-fat diet and to engage in physical activity of moderate

intensity for at least 150 minutes per week, reduced the incidence of T2DM by 58%, while the pharmacological intervention with metformin, an antihyperglycemic agent, reduced the incidence of T2DM by 31%. It cannot be concluded from these two studies, though, that physical activity was the sole cause of the reduction in incidence of T2DM, as physical activity was only one component of the lifestyle interventions. However, Pan et al. (69) did demonstrate that increased levels of physical activity alone can decrease the incidence of T2DM. In a clinical trial of T2DM prevention, subjects with impaired glucose tolerance were randomized into an exercise only, diet only, diet plus exercise, or control group. Subjects in the exercise group were encouraged to increase daily leisure physical activity by one unit, which was generally comparable to a 20-minute brisk walk daily. The cumulative incidence of T2DM at six years was significantly lower in the exercise only group compared to the control group and was comparable to the reduction in incidence in the other groups (exercise = 41%, diet = 44%, diet plus exercise = 46%, control = 68%).

It has also been demonstrated that exercise training alone can have a beneficial effect on insulin resistance and glucose tolerance. Holloszy et al. (37) examined the effect of twelve months of exercise training on insulin resistance and glucose tolerance in men with T2DM, impaired glucose tolerance, or high normal glucose values, while controlling for diet. By the end of the exercise training program, subjects were exercising five days per week for 50-60 minutes at an intensity of 70-90% VO_2max. It was found that prolonged, strenuous and frequent exercise can completely normalize glucose tolerance, as assessed by OGTT, by decreasing insulin resistance in some subjects with mild T2DM and in some

individuals with impaired glucose tolerance. However, it was noted that exercise training appears to be effective in normalizing glucose tolerance only in subjects who still have an adequate capacity to secrete insulin, and in whom insulin resistance is the major cause for abnormal glucose tolerance. It was also noted that subjects did lose on average 4-5 kg of weight due to the exercise training; therefore, weight loss could not be fully ruled out as a mechanism for the decrease in insulin resistance observed. Rogers et al. (72) also examined the effect of exercise on insulin resistance and glucose tolerance in men with T2DM and impaired glucose tolerance, but with one week of intense exercise. Subjects exercised six days in the laboratory for 50-60 minutes at 68 ± 1 % of VO_2max, while one day they walked for 60 minutes at ~60% of maximal heart rate. After seven days of exercise, there was a marked improvement in OGTT glucose and insulin response, demonstrating that exercise alone can result in beneficial changes in insulin resistance and glucose tolerance, as no changes in weight occurred among the subjects. It has been established that after physical training, insulin sensitivity of both skeletal muscle and adipose tissue can improve with or without a change in body composition. Thus, physical activity is an important therapeutic modality for individuals with T2DM. However, the effect of physical activity on insulin sensitivity, and on glucose tolerance, is transient and deteriorates within 72 hours. Therefore, regular physical activity is imperative for type 2 diabetics to sustain improved insulin sensitivity (1).

Physical Activity and the Vitamin D Receptor Gene

Despite the fact that the expression of the genetic predisposition for T2DM is influenced by environmental factors (58), only two studies thus far, both cross-

sectional, have examined physical activity in the association of the VDR gene with glucose levels (66) and with insulin sensitivity (26). Ortlepp et al. (66) tested the influence of the BsmI polymorphism on fasting glucose levels in healthy active men categorized based on physical activity level. In the low physical activity group, defined as regular sports activity of three hours per week or less, fasting glucose was significantly influenced by VDR genotype. Subjects with the BB genotype had significantly higher levels of fasting glucose than subjects with either the Bb or bb genotype; however, in the high physical activity group, defined as regular sports activity of more than three hours per week, the effect of the VDR gene was absent, as there was no significant difference in fasting glucose levels between genotype groups. In a study described previously, Gabel et al. (26) also examined the relationship between the BsmI polymorphism and fasting plasma glucose (FPG), fasting plasma insulin (FPI), insulin sensitivity index (S_I), acute insulin response to glucose (AIR_g), and glucose tolerance (K_g) as a function of physical activity. There was a trend for an interaction effect between the BsmI polymorphism and physical activity level with respect to S_I. S_I increased with increasing physical activity levels in the BB and bb genotype groups, but decreased with increasing physical activity levels in the Bb genotype group. There was no evidence of an interaction effect between the genotype groups and physical activity levels with respect to FPG, FPI, AIR_g, or K_g, though.

Summary and Bases for the Study Hypotheses

In light of the projected increases in the prevalence and national cost of diabetes mellitus, in addition to the morbidity and mortality associated with the disease, the importance of the prevention or delay of T2DM is evident, especially in

those at increased risk for the disease. As the development of T2DM is dependent on both genetic and environmental factors, the identification of those genes conferring an increased risk would aid in the identification of individuals with a heightened susceptibility for the disease. Equally important is the determination of the environmental factors that may attenuate the genetic susceptibility of the disease. The purpose of the present study was to evaluate the association of the FokI and BsmI polymorphisms of the VDR gene with changes in insulin sensitivity as a result of aerobic exercise training in an effort to expand upon the existing literature examining the VDR gene as a potential candidate gene in the development of T2DM. The FokI polymorphism was chosen for study because it is a potentially functional variant within the VDR gene that does not appear to be in linkage disequilibrium with the 3' polymorphisms of the VDR gene (31,92). On the other hand, while the BsmI polymorphism is unlikely to have functional consequences and any disease associations with this variant are likely due to linkage disequilibrium (92), this polymorphism was chosen for study to expand upon the work of Gabel et al. (26). Insulin sensitivity was chosen as the primary outcome variable to expand upon the work of Chiu et al. (20) and Gabel et al. (26), whom also examined the association between insulin sensitivity and the FokI and BsmI polymorphisms, respectively, although via different methods. For the present study, it was hypothesized that f allele carriers would have lower insulin sensitivity than FF homozygotes before training based on the results of Chiu et al. (20). It was hypothesized that b allele carriers would have lower insulin sensitivity than BB homozygotes before training based on the results of Oh et al. (64) and Gabel et al. (26), who examined samples of

subjects most similar to those of the present study. All genotype groups were expected to increase insulin sensitivity with training; however, f allele carriers and b allele carriers were hypothesized to increase insulin sensitivity to a greater extent because of their expected lower initial values for insulin sensitivity compared to FF homozygotes and BB homozygotes, respectively.

APPENDIX E: HUMAN SUBJECTS APPROVAL AND CONSENT FORMS

Reference: IRB HSR Identification Number 04-0342

July 12, 2004

MEMORANDUM

Notice of Results of Final Review by IRB on HSR Application

TO: Dr. James Hagberg
Ms. Amanda J. Harne
Department of Kinesiology

FROM: Dr. Phylis Moser-Veillon, Co-Chairperson
Dr. Marc Rogers, Co-Chairperson
Institutional Review Board

PROJECT ENTITLED:
"Influence of Vitamin D Receptor Gene Polymorphisms on Changes in Insulin Sensitivity with Aerobic Exercise Training"

The Institutional Review Board (IRB) concurs with the departmental Human Subjects Review Committee's (HSRC's) preliminary review of the application concerning the above referenced project. The IRB has approved the application and the research involving human subjects described therein. We ask that any future communications with our office regarding this research reference the IRB HSR identification number indicated above.

We also ask that you not make any changes to the approved protocol without first notifying and obtaining the approval of the IRB. Also, please report any deviations from the approved protocol to the Chairperson of your departmental HSRC. If you have any questions or concerns, please do not hesitate to contact us at irb@deans.umd.edu. Thank you.

ADDITIONAL INFORMATION REGARDING IRB/HSRC APPROVALS

EXPIRATION OF IRB APPROVAL-Approval of non-exempt projects expires one year after the official date of IRB approval; approval of exempt projects expires three years after that date. If you expect to be collecting or analyzing data after the expiration of IRB approval, please contact the HSRC Chairperson in your department about submitting a renewal application. **(PLEASE NOTE: If you are not collecting data from human subjects and any on-going data analysis does not increase the risk to subjects, a renewal application would not be necessary.)**

STUDENT RESEARCHERS – Unless otherwise requested, the IRB will send copies of approval paperwork to the supervising faculty researcher (or advisor) of a project. We ask that such persons pass on that paperwork or a copy to any student

researchers working on that project. That paperwork may be needed by students in order to apply for graduation. **PLEASE BE ADVISED THAT THE IRB MAY NOT BE ABLE TO PROVIDE COPIES OF THAT PAPERWORK, particularly if several years have passed since the date of the original approval.**

Enclosures (where appropriate), will include stamped copy of informed consent forms included in application and any copies of the application not needed by the IRB; copies of this memorandum and any consent forms to be sent to the Chairperson of the Human Subjects Review Committee

CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

Project Title: **APO E genotype and HDL Changes with Exercise Training**

I state that I am over 18 years of age and wish to participate in a program of research being conducted by Dr. James Hagberg in the Department of Kinesiology, University of Maryland.

The purpose of this study is to determine the role that genetics may play in determining how my blood cholesterol levels change with exercise training.

I already completed a telephone interview that determined that I am not physically active, am 50 - 75 years of age, not a diabetic or have controlled diabetes, not taking cholesterol-lowering medications, have normal blood pressure or high blood pressure controlled on medications not affecting my cholesterol levels, have no evidence of lung disease, have an appropriate body weight for my height, and have no other medical problems that would keep me from exercising vigorously. Furthermore, if I am a woman, I must be postmenopausal, defined as no menstrual cycles for at least the last 2 years. I understand that if I am a woman and change my hormone replacement therapy regimen during the study, my participation in the study will be terminated. I also understand that if I have a prior history of ulcers or bleeding disorders, I will be excluded from one test that is part of this study. I also understand that I must have somewhat abnormal levels of cholesterol to enter the study.

I understand that I will complete one orientation and two screening visits. The orientation session will present all aspects of the study and my written informed consent will be provided after all of my questions have been answered. For my first Screening visit, I will report to the laboratory in the morning after an overnight fast and a blood sample will be drawn for blood chemistries and blood cholesterol levels. I understand that I may be excluded from the study if this initial blood sample shows elevated levels of glucose in my blood. I understand that a part of this blood sample will be used to obtain my DNA. A blood sample will also be drawn 2 hours after I drink a sugar solution. I understand that a total of 3 tablespoons of blood will be drawn during this visit. I understand that I will be excluded from the study at this point if I have low cholesterol levels, high triglyceride levels, a low red blood cell count, evidence of kidney or liver disease, or evidence of diabetes. I understand that if I remain qualified to this point, on my second Screening Visit I will undergo a treadmill exercise test to determine if I have heart disease. A physical examination will precede the exercise test. I will then complete a test on an exercise treadmill where the treadmill speed and grade will increase every 3 minutes until I cannot continue or symptoms of heart disease develop. Blood pressure, heart rate, and ECG will be recorded before, during, and after the test. I understand that I will be excluded from the study at this point if I have evidence of heart disease.

I understand that if I meet all of these requirements to enter the study, I will undergo 6-8 weeks of instruction in the principles of an American Heart Association low-fat diet and must follow this diet for the remainder of this study. After this I will undergo Baseline Testing that includes the following tests that will be completed in 5

testing sessions. I will have my blood drawn on 2 or 3 occasions from a vein in my arm in the morning after an overnight fast to measure my cholesterol levels and to assess my immune system. I understand that a maximum of 2 tablespoons of blood will be drawn during these visits. I understand that I will also undergo a second exercise test on a treadmill to measure my cardiovascular fitness. This test will start at 70% of the highest heart rate achieved on my first exercise test and the treadmill grade will increase by 2% every 2 minutes. Blood pressure, heart rate, and ECG will be monitored before, during, and after the test. The test will be stopped when I can no longer continue. During this test I will have a noseclip on my nose and I will breathe through a mouthpiece so that the air that I breathe out can be analyzed. I also understand that my dietary habits will be measured by having me record for 7 days all of the food items that I eat. I understand that on another morning after an overnight fast I will have blood samples drawn before and for 3 hours after I drink a glucose solution to assess my risk of developing diabetes; I will also have additional blood drawn prior to this test that will be frozen for future studies that relate directly to the goals of the present study. I understand that 5 tablespoons of blood will be drawn during this visit. I understand that on another occasion after an overnight fast, I will have blood samples drawn from a line (catheter) in my arm before and for 4 hours after drinking approximately 1 – 2 cups of a high-fat liquid meal. The high-fat meal is made of heavy whipping cream with small amounts of chocolate, sugar, and powdered milk and tastes similar to a rich chocolate shake. I understand that 10 tablespoons of blood will be drawn during this test and will be used to measure how my body absorbs and uses fat from a meal and how my blood clotting, and substances that affect hunger are affected by a fat meal. Before and after I drink the high-fat meal, I understand that I will breath through a mouthpiece while my nose is closed-off with a nose clip and the air that I breath out will be collected and used to determine how much fat I use for energy while sitting at rest. I understand that these tests will be done at the University of Maryland College Park.

I understand that in the morning after an overnight fast I will have blood samples drawn to assess my cholesterol levels and blood clotting system. I will then have a substance that temporarily stops blood from clotting injected into my arm vein. Blood samples are drawn 10 minutes later for measurement of chemicals that affect blood cholesterol levels. I understand that if I have a prior history of ulcers or bleeding disorders I will not undergo this test. I understand that I will remain in the laboratory for 2 - 3 hours after this test with pressure on the site where blood samples were drawn to make sure that all bleeding is stopped. I understand that 4 tablespoons of blood will be drawn during this visit. I understand that how much fat and muscle I have will be measured using x-rays while I lie quietly on a table for 15 to 30 minutes. I understand that the amount of fat I have around my waist will be measured with a CAT scan while I lie quietly on a table. I also understand that these last 3 tests will be done at the VA Medical Center in Baltimore.

I understand that during this Baseline Testing a total of 21 tablespoons of blood will be drawn, which is about two-thirds of the amount of blood given when donating blood.

I understand that after completing this testing, for 6 months I will complete 3 exercise sessions each week supervised by study personnel. I understand that I will be instructed on appropriate warmup and stretching exercises to perform prior to each exercise training session. I will be taught to measure my heart rate and to use heart rate monitors to control how hard I am exercising. The first training sessions will consist of 20 minutes of light exercise. The amount of exercise and how hard I exercise will increase gradually until I am completing 40 minutes of moderate intensity exercise every session. Exercise modes include walk/jogging, stairstepping, and cycle, cross-country ski, and rowing ergometry. I will be asked to add a 45-60 minute walk to my exercise program on weekends after the first 10 weeks of the exercise program. I understand that some of the supervised exercise sessions may be done outside of the exercise facility, but still under the direct supervision of study personnel. I understand that if I lose more weight than expected from the exercise, I will be counseled by a dietitian against restricting how much food I eat. I will also be asked to complete food records during the exercise training program and if major dietary changes have occurred, I will also be counseled by a dietitian to resume my original dietary habits.

I understand that after completing 6 months of exercise training, I will have everything reevaluated that was measured before I began the exercise program. I understand that during this testing a maximum of 21 tablespoons of blood will be drawn, which is about two-thirds of the amount of blood given when donating blood.

I understand that if I qualify for this study that my DNA will be isolated from my blood and analyzed at a number of sites for differences in DNA that may affect how my cholesterol levels change with exercise training. I understand that some of my DNA will also be frozen for future studies. However, these studies can only analyze my DNA at sites that might affect how my cholesterol levels, glucose and insulin levels, bone density, body composition, immunology (disease-fighting), and cardiovascular blood clotting systems change with exercise training.

All information collected in this study is confidential, and my name will not be identified at any time. I understand that my DNA (genetic material) will be sent to laboratories at the University of Pittsburgh and the University of Maryland at Baltimore School of Medicine that are part of this study. I understand, however, that my DNA samples sent to the University of Pittsburgh and the University of Maryland at Baltimore will be identified only by a numeric code. I understand that my coded blood samples are also sent to the University of Florida and to a company in North Carolina to measure compounds in my blood that relate to blood cholesterol levels and cardiovascular disease risk. I understand that only investigators at the University of Maryland College Park will know whose name is associated with each coded number. I further understand that the list of names and codes will be retained at the University of Maryland for up to 25 years.

I understand the following risks are associated with my participation in this study. (1) The risk of maximal exercise testing is approximately 1 nonfatal event in 10,000 tests and 1 fatal cardiac event in 70,000 tests. Risks will be minimized by having the test administered by a physician and personnel trained in such tests and emergency procedures. I will be screened with a resting ECG and a physical examination prior to

this test. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all testing sessions. (2) There is minimal risk of bruising and infection associated with blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The risk of the body composition testing is the exposure to X-rays. The amount of x-ray exposure for this test is the same as that occurring during 30 minutes of any activity outside in the sun. (4) The risk associated with the test requiring the injection into an arm vein of a substance that temporarily stops blood clotting is bleeding. This risk will be minimized by excluding persons with bleeding disorders, peptic ulcers, or other blood disorders from the study. The risk is further minimized by placing a pressure bandage on the intravenous access site after the blood sampling and observing the subject for 2-3 hours after the injection. (5) The risk associated with the CAT scan to measure abdominal fat is the exposure to x-rays. The x-ray exposure is less than the maximum radiation dose individuals are permitted to be exposed to each year in their occupation. (6) The risks associated with the blood clotting and immune system studies are those related to blood drawing as listed above. (7) The risks associated with the oral glucose tolerance test and the high-fat meal test are those associated with blood drawing, the possibility of having low blood sugar levels at the end of the test, and the possibility of having an upset stomach, primarily a stomach ache, after drinking the glucose and/or high-fat meals. The risk of low blood sugar levels at the end of the test will be minimized by providing you with a drink and small snack. (8) The risk of exercise training is the possibility of a heart attack or other cardiovascular event. A large physical activity center reported that 1 nonfatal cardiovascular event occurred in 1.7 million walk/jogging miles. These risks will be minimized because I will undergo a cardiovascular evaluation before beginning exercise training. Exercise sessions will be supervised by experienced personnel trained in emergency procedures. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all supervised exercise training sessions. Two study personnel will supervise the outside exercise sessions done at the University of Maryland, College Park though no emergency equipment will be directly available during these sessions. (9) There are no risks associated with the genetic testing because no results of these tests will be given to the participants. This has to be the case because the genetic results are not from clinically-approved laboratories.

I understand that this study is not designed to help me personally, but may help the investigators to determine who exercise might benefit the most. I understand that I will be provided with my study results and they can be sent to my physician if I request this in writing. I understand that these results are not to be used for clinical diagnostic purposes and that I will not receive the results of my genetic testing. I understand that I am free to ask questions or to withdraw from participation at any time without penalty. I understand that I will be paid $50 for completing Baseline Testing after the dietary stabilization period. I also understand that I will be paid another $50 for completing 3 months of exercise training and another $100, for a total of $200, for completing 6 months of exercise training and all final testing, if I complete at least 90% of my exercise training and testing sessions. I understand that if my participation in the study has to be terminated because I change my hormone replacement therapy regimen, I will

only be paid for the portion of the study that I have already completed, that is, which of the stages above that I have completed.

In the event of a physical injury resulting from participation in this study, I understand that immediate medical attention is available at the Washington Adventist Hospital or the Baltimore VA Medical Center. However, I understand that the University of Maryland does not provide any medical or hospitalization coverage for participants in this research study nor will the University of Maryland provide any compensation for any injury sustained as a result of participation in this research study except as required by law.

Principal Investigator: James Hagberg, PhD. Department of Kinesiology. HLHP Building. University of Maryland, College Park, MD 20742-2611, telephone 301-405-2487.

_____	_____
Subject's signature	Date
_____	_____
Witness	Date
_____	_____
Investigator	Date

CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

Project Title: **ACE genotype, blood pressure, and exercise training in hypertensives**

I state that I am over 18 years of age and wish to participate in a program of research being conducted by Dr. James Hagberg in the Department of Kinesiology, University of Maryland.

The purpose of this study is to determine the role that genetics may play in determining how my blood pressure changes with exercise training. This research project will require visits to University of Maryland College Park and the Baltimore VA Medical Center. The specific tests, their requirements, and the time commitments are described below.

I already completed a telephone interview that determined that I am not physically active, am 50 - 75 years of age, not a diabetic, have no evidence of lung disease, have an appropriate body weight for my height, and have no other medical problems that would keep me from exercising vigorously. It is also probable that I have a blood pressure that is in the Prehypertensive or Stage 1 hypertension range (Systolic blood pressure: 120 –159; Diastolic blood pressure: 80 – 99). Furthermore, if I am a woman, I must be postmenopausal, defined as no menstrual cycles for at least the last 2 years. I understand that if I am a woman and change my hormone replacement therapy regimen during the study, my participation in the study will be terminated.

The flow chart on the next page indicates the different testing sessions and time required by this study. I understand that if I qualify and complete this study my total involvement will last approximately 9 months. Two of the testing visits are performed at the Baltimore VA Medical Center. I understand that I will also be asked to sign University of Maryland Baltimore consent from for the tests conducted in Baltimore.

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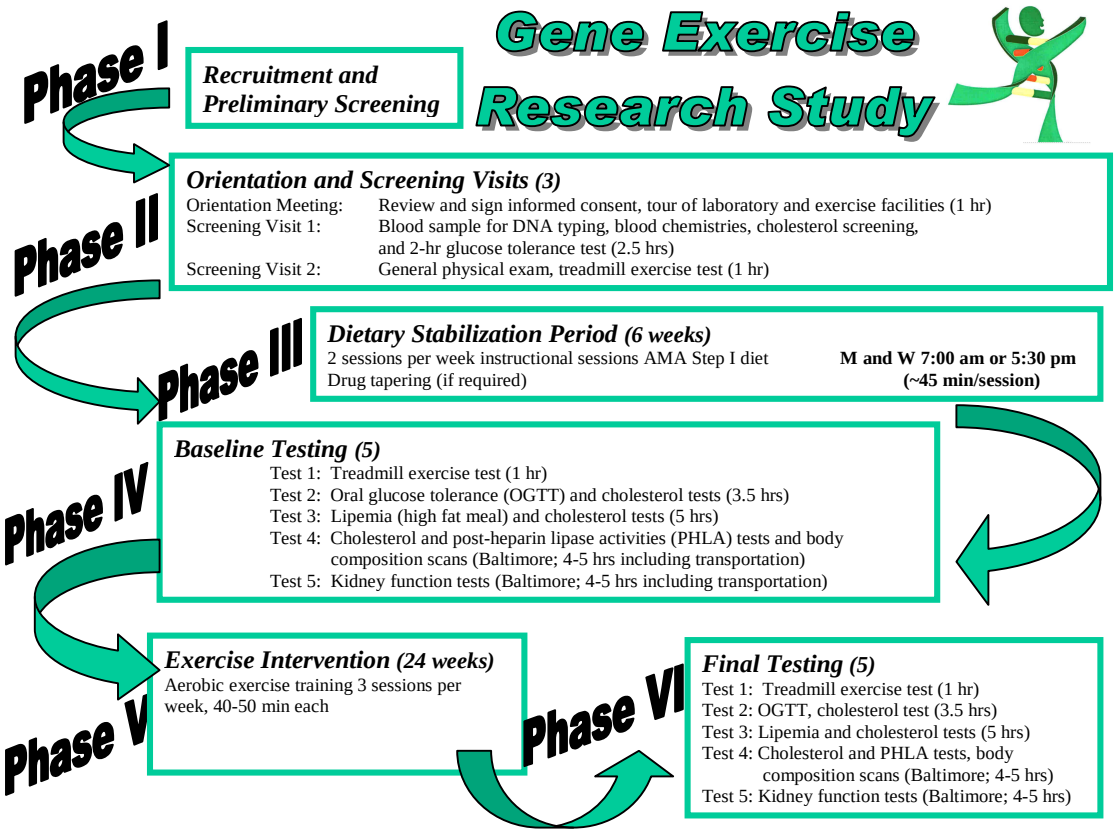
Gene Exercise Research Study
Phase I
Recruitment and Preliminary Screening
Phase II
Orientation and Screening Visits (3)
Orientation Meeting: Review and sign informed consent, tour of laboratory and exercise facilities (1 hr)
Screening Visit 1: Blood sample for DNA typing, blood chemistries, cholesterol screening, and 2-hr glucose tolerance test (2.5 hrs)
Screening Visit 2: General physical exam, treadmill exercise test (1 hr)
Phase III
Dietary Stabilization Period (6 weeks)
2 sessions per week instructional sessions AMA Step I diet
Drug tapering (if required)
M and W 7:00 am or 5:30 pm (~45 min/session)
Phase IV
Baseline Testing (5)
Test 1: Treadmill exercise test (1 hr)
Test 2: Oral glucose tolerance (OGTT) and cholesterol tests (3.5 hrs)
Test 3: Lipemia (high fat meal) and cholesterol tests (5 hrs)
Test 4: Cholesterol and post-heparin lipase activities (PHLA) tests and body composition scans (Baltimore; 4-5 hrs including transportation)
Test 5: Kidney function tests (Baltimore; 4-5 hrs including transportation)
Phase V
Exercise Intervention (24 weeks)
Aerobic exercise training 3 sessions per week, 40-50 min each
Phase VI
Final Testing (5)
Test 1: Treadmill exercise test (1 hr)
Test 2: OGTT, cholesterol test (3.5 hrs)
Test 3: Lipemia and cholesterol tests (5 hrs)
Test 4: Cholesterol and PHLA tests, body composition scans (Baltimore; 4-5 hrs)
Test 5: Kidney function tests (Baltimore; 4-5 hrs)

I understand that I will complete 1 Orientation and 2 initial screening visits. For my first visit, the study will be explained to me, my medical history will be reviewed, and I will provide my written informed consent. This visit will last about 60 minutes. On my first screening visit, I will report to the laboratory in the morning after an overnight fast and a blood sample will be drawn for blood chemistries and for isolation of my DNA. I will have my height, weight, and blood pressure measured. I understand that I may be excluded from the study if this initial blood sample shows elevated levels of glucose or creatinine in my blood. A blood sample will also be drawn 2 hours after I drink a sugar solution. This visit will last about 2 ½ hours. I understand that a total of 3 tablespoons of blood will be drawn during this visit. I understand that I will be excluded from the study at this point if I have low red blood cell count, evidence of kidney or liver disease, evidence of diabetes, or if my blood pressure is too high or too low.

I understand that if I remain qualified to this point, I will undergo a treadmill exercise test to determine if I have heart disease. A physical examination will precede the exercise test. I will have my resting blood pressure measured. I will then complete a test on an exercise treadmill where the treadmill speed and grade will increase every 3 minutes until I cannot continue or symptoms of heart disease develop. Blood pressure, heart rate, and electrocardiogram (electrical activity of my heart) will be recorded before, during, and after the test. During this test I will have a noseclip on my nose and I will breathe through a mouthpiece so that the air that I breathe out can be analyzed. I understand that this visit will last about 1 hour and that I will be excluded from the study at this point if I have evidence of heart disease.

I understand that if I meet these requirements to enter the study and if I am taking medications to control my blood pressure, I give my permission for my private physician to be contacted to obtain their approval for me to stop taking these medications for the remainder of this study. I understand that I will be slowly withdrawn from these medications according to the plan my physician provides and that my blood pressure will be measured weekly for the remainder of the study. I also understand that if my blood pressure is too high (Systolic blood pressure: >159; Diastolic blood pressured: >99) for three consecutive weeks at any time during the study, I will be excluded from further participation in the study and referred back to my physician. If I am in the exercise training portion of the study, I understand that if this happens I will complete all Final Testing (see below) and then be referred back to my physician. I also understand that a physician from the University of Maryland School of Medicine is directly involved in this study and that he can be contacted for any medical questions, but only as they concern my involvement in this study.

I understand that if I meet these requirements to enter the study, I will undergo 6 weeks of instruction in the principles of an American Heart Association low-fat and low salt diet and must follow this diet for the remainder of this study. This program consists of two 40 minute classes each week for the 6 week duration of the program. During the final 3 weeks of this dietary program, I understand that I will have my blood pressure measured weekly for 3 weeks. I understand that my blood pressure must average in the range of 120- 159 for systolic or 80 – 99 for diastolic

blood pressure for me to continue in the study.

After this I will undergo Baseline Testing that includes the following tests that will be completed in 7 testing sessions (5 at the University of Maryland, College Park and 2 at the Baltimore VA Medical Center). I will have blood drawn on 2 occasions from a vein in my arm in the morning after an overnight fast to measure my cholesterol levels; these visits will each last about 20 minutes. I understand that a maximum of 2 tablespoons of blood will be drawn during these visits. I understand that I will also undergo a second exercise test on a treadmill to measure my cardiovascular fitness. This test will start at 70% of the highest heart rate achieved on my first exercise test and the treadmill grade will increase by 2% every 2 minutes. Blood pressure, heart rate, and electrocardiogram will be monitored before, during, and after the test. The test will be stopped when I can no longer continue. During this test I will have a noseclip on my nose and I will breathe through a mouthpiece so that the air that I breathe out can be analyzed. I understand that this visit will last about 1 hour. I also understand that my dietary habits will be measured by having me record for 7 days all of the food items that I eat. I understand that I will collect my urine for 24 hours in a container that must be refrigerated so that the amount of salt I eat in my diet can be measured; I also understand that my blood pressure will be monitored throughout this 24 hour period with a cuff around my upper arm and a "Walkman-size" controller worn at my waist. I also understand that I will undergo a 3 hour glucose tolerance test where I will come to the laboratory in the morning after an overnight fast, have a small catheter inserted in an arm vein for blood sampling, and have blood samples drawn before and for every 30 minutes after I drink a glucose solution. Additional samples will be drawn before this test to measure hormone levels in my blood that affect my blood pressure, immunological (disease-fighting), and blood clotting systems. I understand that a maximum of 7 tablespoons of blood will be drawn for this portion of the study. I understand that on another occasion after an overnight fast, I will have blood samples drawn before and every 30 minutes for 4 hours after drinking 1 – 2 cups of a high-fat liquid meal. These blood samples also will be drawn through a small catheter inserted into my arm vein. The high-fat meal is made of heavy whipping cream with small amounts of chocolate, sugar, and powdered milk and tastes like a rich chocolate shake. I understand that 10 tablespoons of blood will be drawn during this test and will be used to measure how my body absorbs and uses fat from a meal and how my blood clotting, and substances that affect hunger are affected by a fat meal. Before and after I drink the high-fat meal, I understand that I will breath through a mouthpiece while my nose is closed-off with a nose clip and the air that I breath out will be collected and used to determine how much fat I use for energy while sitting at rest. I also understand that I will complete a test that takes about 1 hour to measure the blood flow in my arm at rest and immediately after stopping my arm blood flow for 5 minutes with the use of a blood pressure cuff. I understand that all of these tests listed above will be done at the University of Maryland College Park.

 Initials _____

I understand that on a visit to Baltimore on a separate day, I will have my kidney function measured at the Clinical Research Unit, Division of Nephrology, University of Maryland at Baltimore after an overnight fast. Before the test, I will drink 17 ounces of water over a 30-minute period. A Registered Nurse will then insert a small needle into veins in both of my arms. One line will be used to give the study medications and the other will be used to draw blood samples. Before the study drugs are given, I will provide a urine sample and a 0.7 ounce blood sample. During the test I will remain in a seated position except for when I provide urine samples. Next, I will receive the study medications, para-aminohippuarte and iothalamate, which are markers used for estimating kidney function. Para-aminohippurate (5mg/kg body weight) and iothalamate (434 mg) will be given over five minutes. Then I will receive an additional small dose of para-aminohippurate and iothalamate by a slow, continuous infusion so that I will have the necessary amounts in my blood. Four blood samples (~0.7 ounces) will be drawn over the next 2½ hours and I will be asked to collect my urine every ½ hour for the next 2½ hours. This test will take approximately 3 hours. A total of 1.4 ounces of blood will be drawn during this test. I understand that I will undergo this test twice, once before and once after 6 months of aerobic exercise training. I understand that the amount of fat I have around my waist will be measure with a CAT can while I lie quietly on a table. Another study will be done to measure my total body fat mass and total body muscle mass while I lie quietly on a table. I also understand that if I have elevated blood cholesterol levels, I will have blood samples drawn before and after a substance that temporarily stops blood from clotting is injected into my arm vein. The blood samples will be used to measure chemicals that affect my blood cholesterol levels. A total of 4 tablespoons of blood will be drawn at this visit. I understand I will remain in the VA Medical Center for 2 - 3 hours after this test to make sure that all bleeding is stopped. I also understand that these tests will be done at the VA Medical Center in Baltimore. I understand that each of these visits will require approximately 4 – 5 hours including travel time.

I understand that the maximum total amount of blood that will be drawn during this Screening and Baseline Testing is 28 tablespoons over 2 – 3 months. This is approximately 90% of the amount of blood that is typically drawn during a single blood donation.

I understand that after completing this testing, for 6 months I will complete 3 exercise sessions each week supervised by study personnel. I understand that I will be instructed on appropriate warmup and stretching exercises to perform prior to each exercise training session. I will be taught to measure my heart rate and to use heart rate monitors to control how hard I am exercising. The first training sessions will consist of 20 minutes of light exercise. The amount of exercise and how hard I exercise will increase gradually until I am completing 40 minutes of moderate intensity exercise every session. Exercise modes include walk/jogging, stairstepping, and cycle, cross-country ski, and rowing ergometry. I will be asked to add a 45-60 minute walk to my exercise program on weekends after the first 10 weeks of the exercise program. I understand that this is not designed as a weight loss program and

that if I lose more weight than expected from the amount of exercise that I complete, I will be counseled by a dietitian against restricting how much food I eat. I will also be asked to complete food records during the exercise training program and if major dietary changes have occurred, I will also be counseled by a dietitian to resume my original dietary habits. I understand that I may also be asked to collect my urine for 24 hours during the exercise training portion of the study.

I understand that after completing 6 months of exercise training, I will have everything reevaluated that was measured before I began the exercise program. I understand that during this 4 weeks of Final Testing a maximum of 28 tablespoons of blood will be drawn; this is approximately 90% of the amount of blood that is typically drawn during a single blood donation.

I understand that if I qualify for this study that my DNA will be isolated from my blood and analyzed at a number of sites for differences in DNA that may affect how my blood pressure change with exercise training. I understand that some of my DNA will also be frozen for future studies. However, these studies can only analyze my DNA at sites that might affect how my blood pressure, cholesterol levels, glucose and insulin levels, bone density, body composition, immunology (disease-fighting), and cardiovascular and blood clotting systems change with exercise training.

All information collected in this study is confidential, and my name will not be identified at any time. I understand that my DNA (genetic material) will be sent to collaborating genetics laboratories that are part of this study and that a sample of my DNA will be kept in the University of Maryland Department of Kinesiology laboratories. I also understand that samples of my blood will be sent to other collaborating laboratories for other blood measurements. I understand, however, that my DNA and blood samples sent to these laboratories will be identified only by a numeric code and that only investigators at the University of Maryland College Park will know whose name is associated with each coded number. I further understand that the list of names and codes will be retained at the University of Maryland for up to 25 years.

I understand the following risks are associated with my participation in this study. (1) The risk of maximal exercise testing is approximately 1 nonfatal event in 10,000 tests and 1 fatal cardiac event in 70,000 tests. Risks will be minimized by having the test administered by a physician and personnel trained in such tests and emergency procedures. I will be screened with a resting electrocardiogram and a physical examination prior to this test. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all testing sessions. (2) There is minimal risk of bruising and infection associated with blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The risk of the body composition testing is the exposure to X-rays. The amount of x-ray exposure for this test is the same as that occurring during 30 minutes of any activity outside in the sun. (4) There is some risk associated with the elevated blood pressure that I have and some risk associated with stopping the mediations I take to control my blood pressure. However, a 12-month lifestyle change program including diet and exercise is part of the medical recommendations for blood pressure control for individuals with

levels of blood pressure similar to mine. In addition, I understand that my blood pressure will be monitored weekly and that this exceeds the blood pressure follow-up guidelines recommended for physicians. I also understand that if my blood pressure is too high for three consecutive weeks anytime during the study, my participation in the study will be discontinued and I will be referred back to my private physician. I also understand that a physician associated with this project is available to deal with concerns related to my participation in this study. (5) The risk associated with the CAT scan to measure abdominal fat is the exposure to X-rays. The X-ray exposure is less than the maximum radiation dose individuals are permitted to be exposed to each year in their occupation. (6) There are no risks associated with the 24 hour urine collection. (7) The only risks associated with the measurement of the hormones in my blood that affect my blood pressure are those associated with blood drawing. (8) The risks associated with the glucose tolerance test and the high fat meal are those related again to blood sampling, the possibility that my blood sugar may go too low levels at the end of the test, and the possibility of an upset stomach, primarily a stomach ache, after drinking the glucose or high-fat meal. I understand that I will be given a juice drink and small snack to minimize the chances of my blood glucose levels decreasing too much. (9) There are no risks associated with the genetic testing because I will not be provided with these results. (10) The risk of exercise training is the possibility of a heart attack or other cardiovascular event. A large physical activity center reported that 1 nonfatal cardiovascular event occurred in 1.7 million walk/jogging miles. These risks will be minimized because I will undergo a cardiovascular evaluation before beginning exercise training. Exercise sessions will be supervised by experienced personnel trained in emergency procedures. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all supervised exercise training sessions. (11) If I have elevated blood cholesterol levels, I understand that the risk associated with the test requiring the injection into an arm vein of a substance that temporarily stops blood clotting is bleeding. This risk will be minimized by excluding people with bleeding disorders, peptic ulcers, or other blood disorders from the study. The risk is further minimized by placing a bandage on the intravenous access site after the blood sampling and observing the subject for 2-3 hours. (12) The risks associated with the tests to assess kidney function are low as these are routine clinical tests. The risks are the side effects of the compounds put into my blood to assess kidney function; side effects include nausea, vomiting, facial flush, a generalized feeling of warmth, and allergic reactions. The risks are also those associated with blood drawing. These risks are minimized by administering these tests in a hospital setting with nurses experienced with these methods administering the test, so that if I should experiences these side effects, medical personnel and equipment are readily available to respond and treat these symptoms. (13) The risks associated with the 24 hour blood pressure recording are the possibility of sleep disturbances in about 2% of volunteers. (14) The risks associated with the measurement of the blood flow in my arm are the result of stopping blood flow to the arm for 5 minutes. This causes substantial discomfort that ceases shortly after the blood pressure cuff is removed. I understand that if I can not tolerate the discomfort, this test will be terminated immediately on my request.

 Initials _____

I understand that this study is not designed to help me personally, but may help the investigators to determine whom exercise might benefit the most. I understand that I am free to ask questions or to withdraw from participation at any time without penalty. I understand that I will earn $50 at the completion of Baseline Testing after the dietary stabilization period. I also understand that I will earn another $50 after 3 months of exercise training if I complete at least 90% of my exercise training sessions. I also understand that I will earn another $100 after completing 90% of my training sessions for 6 months and all final testing. I understand that the total amount that I earn will be paid to me at the completion of my participation in the study. I understand that if my participation in the study has to be terminated because I change my hormone replacement therapy regimen, I will only be paid for the portion of the study that I have already completed, that is, which of the stages above that I have completed.

In the event of a physical injury resulting from participation in this study, I understand that immediate medical attention is available at the Washington Adventist Hospital or the Baltimore VA Medical Center. However, I understand that the University of Maryland does not provide any medical or hospitalization coverage for participants in this research study nor will the University of Maryland provide any compensation for any injury sustained as a result of participation in this research study except as required by law.

Principal Investigator: James Hagberg, PhD. Department of Kinesiology. HLHP Building. University of Maryland, College Park, MD 20742-2611, telephone 301-405-2487.

_____	_____
Subject's signature	Date
_____	_____
Witness	Date
_____	_____
Investigator	Date

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